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An Investigation into Formulation and Therapeutic Effectiveness of Nanoparticle Drug Delivery for Select Pharmaceutical Agents

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An Investigation into Formulation and Therapeutic Effectiveness of Nanoparticle Drug Delivery for Select Pharmaceutical Agents

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by

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ABSTRACT

An Investigation into Formulation and Therapeutic Effectiveness of Nanoparticle Drug Delivery for Select Pharmaceutical Agents

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Drug based nanoparticle (NP) formulations have gained considerable attention over the past decade for their use in various drug delivery systems. NPs have been shown to increase bioavailability, decrease side effects of highly toxic drugs, and prolong drug release. Furthermore, polymer based, biodegradable nanodelivery has become increasing popular in the field of NP formulation because of their high degree of compatibility and low rate of toxicity.

Due to their popularity, commercially available polymers such as poly lactic acid (PLA), poly glycolic acid (PGA) and polylactic-co-glycolic acid (PLGA) are commonly used in the development and design of new nano based delivery systems. Nonsteriodal anti-inflammatory drugs (NSAIDs) are commonly used for the treatment of pain and inflammation. NSAIDs such as diclofenac and celecoxib function by blocking cyclooxygenase expression and reducing prostaglandin synthesis. Unfortunately, the pharmacological actions of NSAIDs can lead to the development of several adverse side effects such as gastrointestinal ulceration and bleeding.

The aim of this study was to formulate and optimize diclofenac or celecoxib entrapped polymer NPs using an emulsion-diffusion-evaporation technique. NP formulations were evaluated based on specific formula parameters such as particle size, zeta potential, morphology, and entrapment efficiency. Effects of stabilizer type, stabilizer concentration, centrifugal force, drug amount, and/or emulsifier (lecithin) on nanoparticle characterization were examined for formula optimization.

Results of the formulation studies showed that NPs developed using polylactide-co-glycolide (PLGA) polymers and the stabilizer didodecyldimethylammonium bromide (DMAB) demonstrated enhanced stability, drug entrapment, and reduced particle size. These findings demonstrate an effective method for polymer NP formulation of diclofenac or celecoxib. Furthermore, the results reported herein support a novel method of drug delivery that may function to reduce known adverse effects of these pharmacotherapeutic agents.

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CHAPTER 1

INTRODUCTION

Nanoparticles (NPs) are solid colloidal particles ranging in size from 10 to 1000 nm and contain nanomolecular materials, in which active ingredients are dissolved, entrapped, or encapsulated (1 - 3). NPs are currently gaining popularity in the medical field as an effective means of drug delivery and therapy (Shive and Anderson 1997; Buzea et al 2007; De Jong, and Borm 2008; Cooper and Harirforoosh 2014). Through the use of colloidal based NPs, researchers are effectively creating a drug delivery system that can improve a drug's overall kinetics, dynamics, and targeted release (Cooper et al 2014). Altered drug formulation designs using NP encapsulated drugs offer a safe and effective alternative in pharmacotherapy. New NP formulations have been found to be well suited for a variety of drugs in the biomedical field by offering a higher degree of safety and drug efficacy with regard to disease treatment and the onset of drug associated adverse side effects (Shive and Anderson 1997; Doane and Burda 2012; Cooper et al 2014). Drug loaded NPs have been shown to increase the therapeutic indices of entrapped agents and reduce development of adverse side effects (Shive and Anderson 1997; Uchino et al. 2005; Desai et al. 2008; Li et al. 2011; Vishnu and Roy 2011; Doane and Burda 2012; Cooper et al 2014). Recent studies on chemotherapeutic and antibiotic NP encapsulated drugs have shown potential in accelerating disease treatment and reducing associated adverse complications (Mizumura et al 2001; Uchino et al 2005; Li et al 2011; Vishnu and Roy 2011; Benival and Devarajan 2012; Jain et al 2012; Zhao et al 2013; Cooper et al 2014).

Pharmaceutical agents such as nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used among the general population (Cooper et al 2014). NSAIDs act to block the formation of the inflammatory messenger molecule prostaglandin by inhibiting the cyclooxygenase (COX) enzyme (Simmons 2004; Brater 2006; Dogne 2006; Cooper et al 2014). The most commonly reported side effects of these drugs are gastrointestinal ulcerations, dyspepsia, and diarrhea. Certain cardiovascular side effects such as stroke and myocardial infarction are also common among COX-2-selected inhibitors

such as rofecoxib and celecoxib (Goldberg 1999; Simmons et al 2004; Caldwell et al 2006; Dogne et al 2006).

Evidence suggest that the use of NPs in drug delivery may effectively reduce complications associated with the use of NSAIDs (Mizumura et al 2001; Uchino et al 2005; Desai 2008; Li et al 2011; Vishnu and Roy 2011; Benival and Devarajan 2012; Jain et al 2012; Zhao et al 2013; Cooper et al 2014). In the literature, drug reformulation studies using polymer based NPs have shown positive benefits in regards to drug induced complications as well as reductions in associated adverse events. When applied towards the commonality of NSAID use, and the known efficacy and side effects associated with such drugs, investigation into NP reformulation of these pharmaceutical agents was warranted.

Currently, there are many types of polymers available for polymer based NP formulation. Examples of common polymers employed in NP formulation are poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and poly (lactic-co-glycolic acid) (PLGA) (Cooper et al 2014). Like many NP delivery systems, polymer based NP formulation can act to offset drug release by desorption of bound drug from particle surfaces, erosion of the polymer membrane, and/or drug diffusion (Buzea et al 2007; Dejong and Borm 2008; Cooper et al 2014). The three aforementioned stages at which polymer NPs can release active pharmaceutical agents makes them an ideal choice in attempting to formulate a new and novel drug delivery systems that could act to modify or control drug release and offset the occurrence of drug related adverse events.

In regards to polymer choice, PLA has been shown to exhibit a prolonged rate of degradation and drug release (Cooper et al 2014). In counter to PLA characteristics, PGA is commonly used in formulations were fast hydrolysis and erosion are warranted. As such, in this study focused was placed on the use of PLGA because of its known intermediate rate of hydrolysis and biodegradation in comparison to PLA or PGA. Furthermore, polymeric NP formulations consisting of PLGA can result in minimal or reduced toxicity due to product conversion to the Krebs cycle intermediates lactic acid and

glycolic acid. Because of these favorable characteristics, PLGA was chosen as the primary polymer to be used.

Solvent evaporation is one of the more commonly used formulation techniques for production of polymer based NPs (Savjani et al 2012; Cooper et al 2014, Cooper and Hariroforoosh 2014; Cooper and Harirforoosh 2014). In this study polymer encapsulated pharmaceutical agents were formulated using a previously described oil in water solvent evaporation technique Cooper and Hariroforoosh 2014; Cooper and Harirforoosh 2014). To facilitate NP formulation, an organic phase consisting of polymer and drug dissolved in ethyl acetate was added to an aqueous phase containing a stabilizer. Ethyl acetate has been shown to be effective in the formulation and creation of drug loaded NP delivery systems and was thus chosen as the primary solvent for these formulation studies. The stabilizer didodecydimethylammonium bromide (DMAB) is highly effective at creating positively charged particles (Cooper and Hariroforoosh 2014). In DMAB based formulation studies, these positively charged surface characteristics have been shown to impede particle agglomeration and enhance system stability, making them an ideal stabilizer of choice for new NP formulation analysis (Cooper and Hariroforoosh 2014; Cooper and Harirforoosh 2014). A second stabilizer, poly vinyl alcohol (PVA), was also chosen as a comparison model for stabilizer effects in NP formulation. PVA has been extensively used in various formulation designs. Due to PVA's extensive history in drug delivery and formulation, it was chosen for comparative stabilizer analysis during formula optimization techniques.

The overall aim of this research was to formulate, develop and optimize a polymeric NP loaded drug delivery system. The results shown herein outlines development of this new delivery systems for the select pharmaceutical agents, diclofenac and celecoxib.

As a whole, formulation aspects of this study were directed at elucidating effects of polymer type, drug and stabilizer concentration, and emulsifier effects on overall drug loaded NP characteristics. Commonly reported NP characteristics including particle size, zeta potential, drug entrapment, morphology, and *in vitro* release rates of various NSAID reformulations were determined. The

examination of particle size and zeta potential was performed using a NICOMP Zeta Sizer (Particle Sizing Systems, Port Richey, FL, USA). *In vitro* drug release and drug entrapment investigations were carried out using ultra violet spectrometry (Eppendorf Biophotometer, Hauppauge, NY, USA). Drug loaded NP morphology was examined via transmission electron microscopy (Tecnai Philips Transmission Electron Microscope; FEI, Hillsboro, Oregon, USA).

All experiments outlined above were used to study and elucidate the most effective and efficient means of developing polymer drug encapsulated NPs of diclofenac or celecoxib. Major pit-falls and difficulties were not expected during the formulation process. However, a trial and error process was required to identify peak formulation parameters for polymer drug formulation. For example, variations in stabilizer concentration can lead to altered particle characteristics. If a certain concentration of stabilizer resulted in unsatisfactory particle characteristics (i.e. low zeta potential, reduced encapsulation), formulations utilizing higher stabilizer concentrations were examined in an attempt to achieve peak particle parameters. Other optimization techniques were also employed in an effort to reach ideal particle characteristics.

CHAPTER 2

Nanoparticles in Drug Delivery: Mechanism of Action, Formulation, and Clinical Application Towards Reduction in Drug Associated Nephrotoxicity.

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Abstract

Introduction: Over the past few decades, nanoparticles (NPs) have gained immeasurable interest in the field of drug delivery. Various NP formulations have been disseminated in drug development in an attempt to increase efficiency, safety, and tolerability of incorporated drugs. In this context, NP formulations that increase solubility, control release, and/or affect the *in vivo* disposition of drugs, were developed to improve the pharmacokinetic and pharmacodynamic properties of encapsulated drugs.
Areas Covered: In this article, important properties related to NP function such as particle charge, size, and shape are disseminated. Also, the current understanding of how NP characteristics affect particle uptake and targeted delivery is elucidated. Selected NP systems currently used in delivery of drugs in biological systems and their production methods are discussed as well. Emphasis is placed on current NP formulations that are shown to reduce drug induced adverse renal complications.
Expert Opinion: Formulation designs utilizing NP encapsulated drugs offer alternative pharmacotherapy options with improved safety profile for current and emerging drugs. Nanoparticles have been shown to increase the therapeutic index of several entrapped drugs mostly by decreasing drug localization and side effects on organs. Recent studies on NP encapsulated chemotherapeutic and antibiotic medications show enhanced therapeutic outcomes by altering drug degradation, increasing systemic circulation, and/or enhancing cellular uptake. They may also reduce the distribution of encapsulated drugs into kidneys and attenuate drug-associated adverse renal complications. The usefulness of NP formulation in reducing the nephrotoxicity of nonsteroidal anti-inflammatory drugs is an under explored territory that deserves more attention.

Keywords: nanoparticle, formulation, liposome, polymer, bioavailability, biodegradation, nephrotoxicity, NSAIDs

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Article highlights.

‡Research into the functional aspects of particle science has led to increased interest in NP based drug delivery for enhancement of the therapeutic effects of selected drugs.

‡Characteristic aspects of NPs enables the use of drug loaded NPs for improved targeting and cellular uptake by altering particle properties such as drug lipophilicity, cell adhesion, receptor binding, and endocytosis based drug uptake.

‡NP formulation can be achieved through differing methods of development based on drug, particle type, characteristics, and delivery form. Several methods are employed in industry and academia for successful development of liposomal and polymer based NP delivery systems. Solvent evaporation techniques are commonly employed in both industry and academia for successful development of a wide range of NP systems.

‡Altered degradation rates can lead to functional differences in drug effects and systemic exposure. NPs have successfully altered pharmacokinetic and pharmacodynamics properties of selected drugs, leading to the development of sustained drug delivery systems that work to increase patient compliance and reduce the onset of drug induced side effects.

‡Nephrotoxic side effects are common for a number of prescribed medications. NP science has shown enhanced beneficial aspects of known nephrotoxic drugs utilizing NP drug delivery. *In vivo, in vitro*, and clinical trials have demonstrated improved renal effects in paclitaxel, amphotericin B, and cyclosporine based drug regimens. These and other findings have led to development of several FDA approved formulations showing improved clinical efficacy and minimization of common drug side effects, suggesting further research into the effect of NP formulation for improved clinical efficacy of other known nephrotoxic drugs such as NSAIDs.

This box summarizes key points contained in the article

Introduction

Nanoparticles (NPs) are small particles that range in size from 10 nm to 1000 nm [1]. They contain nanomolecular materials in which active ingredients are dissolved, entrapped, and/or encapsulated. At present, NPs can be found in hundreds of different consumer products ranging from sunscreen and air conditioners to processed food supplies [2]. Over the past few decades, NPs have gained recognition in the medical field as an effective means of drug delivery and therapy [3].

Nanometer size range can effectively alter a substance's physical, chemical, and biological properties [4]. On the other hand, NP surface properties can be altered through the utilization of various substances such as polysaccharides, proteins, and polymers [5]. The size and surface properties of NPs can be optimized for given drugs. This opportunity allows the re-formulation of many different therapeutics to new pharmaceutical products with a potential for increased activity and reduced toxicity [6].

Active ingredients can be encapsulated within NPs of differing functionality and chemical structure. This allows for the formulation of a multitude of NP based delivery systems [7] that can exhibit unique properties related to their intended use. The ultimate objective of research in this area is to design a suitable NP, i.e., NPs of appropriate size, chemical structure, and surface characteristics, that can encapsulate clinically relevant amounts of an intended drug with enhanced drug kinetics and dynamics in the biological system [4]. The choice of materials to be used for the production of NP formulations is dependent on the desired functionality one wishes to achieve with respect to specific physicochemical characteristics of a drug and its intended pharmacological activity [8]. In this manuscript, we discuss the function and preparation of selected NP systems used in biomedical research. Emphasis is then placed on the role of NP formulations in reducing renal side effects of commonly known nephrotoxic drugs.

Nanoparticle Mechanism of Action

NP drug encapsulation offers several advantages in creating effective means of drug delivery and localization. NP traits such as particle size, surface charge, and shape play important roles in creating effective NP delivery systems that function through a variety of mechanisms.

1.1. Effect of Particle Size

Particle size can affect the efficiency, biodistribution, and cellular uptake of various NP systems [9]. It is thought that size parameters can play significant roles in the determination of cell interaction and adhesion for various NPs. Size can also play an important part in degradation and elimination processes of NPs. In certain NP systems, the primary aim is to avoid the reticuloendothelia system that targets

foreign bodies for degradation. Avoidance of this system results in an increase of total blood circulation time and bioavailability. As such, it is important to note that nanoparticle size has been directly correlated with clearance rate. As the size of NPs increase, the rate of clearance increases as well. It has been shown that NPs with hydrophilic surfaces exhibiting a particle size less than 100 nm can effectively avoid the mononuclear phagocytic system (MPS) [10]. MPS is a critical element in physiological systems for the elimination of foreign substances. Blood serum contains opsonin proteins which can efficiently bind to larger NPs and tag them for MPS degradation [11]. NPs that obtain small particle diameter and hydrophilic properties can avoid opsinization and MPS degradation, thus enhancing total blood circulation time [10, 11]. Nanometric particles can undergo extensive cellular uptake in comparison to micrometric particles [12]. In a study conducted by Desai *et al.*, it was shown that nanoparticle uptake of an *in situ* rat intestinal loop model demonstrated 15 – 250 fold increases in cellular uptake when compared to larger microparticles [13]. Other studies have shown positive particle size influence in regards to cellular and tissue uptake. In these studies, it was demonstrated that only nano sized particles underwent extensive uptake in comparisons to their larger, microparticle counterparts [14]. Findings such as these demonstrate the influence and importance of particle size for intracellular delivery of various active agents used within the realm of drug therapeutics.

1.2. Effect of Particle Charge

NP charge plays a critical role in the action and efficacy of NP delivery to and through cellular membranes [15]. Stability of an NP system is facilitated through the degree of surface charge present on NPs [16]. A highly charged system undergoes a much larger degree of repulsion between like charged particles. This net repulsive force acts to stabilize and prevent NP aggregation. NPs formulated with more pronounced surface charges have been shown to stabilize NP suspension and prevent particle aggregation. Surface charge characteristics can determine NP degree of absorption as it has been found that NPs with highly positive charges can interact with the anionic polyelectrolyte properties of mucus, resulting in enhanced muco-adhesion and retention of NPs within the mucus layer. Investigational studies performed with PLGA and poly vinyl alcohol formulated NPs have resulted in NPs with highly negative surface charges, which may explain their high reabsorption ability through natural pathways. Many cellular membranes are negatively charged. NPs formulated with known anionic polymers or surfactants will be presented with higher net negative surface charge. This increased negative surface charge will result in repulsion of the NP when it comes into the vicinity of cell membranes. As a result of this repulsive force, cellular uptake becomes difficult and cellular adhesion is reduced. Positively

charged nanoparticles experience opposite effects. The cationic NP facilitates membrane attraction and adhesion, which creates favorable properties for cellular uptake via endocytosis or other mechanisms.

1.3. Effect of Particle Shape

In recent years, research has revealed that particle shape may have fundamental effects on the biological properties of NPs [17]. In a study conducted by Geng *et al.,* it was found that polymer micelles of shorter stature showed an increased total blood circulation time following intravenous (IV) injection [18]. When compared to longer micelles, shorter spheres also underwent a higher degree of cell uptake and effectively delivered the drug, paclitaxel, to targeted tumor cells. Another study found that the length of NP inversely influenced cellular adhesion. In that study it was found that as particle length increased the subsequent binding of NPs decreased, suggesting that attachment and adhesion is a function of cellular length [19]. These studies suggest the importance of NP shape in therapeutic outcomes in relations to drug design and delivery. In NP development, characterization and design must not only pertain to particle size or surface charge, rational design and analysis of shape effects on targeted NP outcomes must be dually considered during NP drug delivery research.

1.4. Cell Targeting

Many biological targets for nanomedicines are large complex molecules such as membrane receptors [20]. Biological processes are initiated through polyvalent interaction between these targeted receptors and its appropriate ligand. Many NP formulations have been developed that largely overlook NP valence capacitance and receptor interactions. However, some formulations such as dendrimer and polymer based NPs have been documented to function through polyanionic receptor mediated targeting [15]. Dendrimer based NP systems have demonstrated targeted viral and cellular interactions via polyvalent interactions with varying surface proteins [20]. Folate formulated polymer based NPs have been shown to bind to overexpressed folate receptors common to tumor cells and initiate cellular entry [15]. Other polymer formulated NPs have shown specificity for caveolae and clatherin proteins resulting in endocytosis uptake via differing target mechanisms [21]. Polymeric micelles have demonstrated the ability to target cancer cells and initiate cellular uptake while avoiding excess uptake in normal epithelial cells [15]. This difference in cell type uptake is thought to be a result of NP differentiation of endocytosis mechanisms common to each cell type [22]. Carcinogenic cellular uptake is initiated through caveolae mediated endocytosis which is absent in normal cell lines. The caveolae targeting capacitance of polymeric micelles enables drug uptake into cancerous cell lines while avoiding drug uptake in

normal cells. As a result, cytotoxic drugs can be formulated in polymer based micelles for cancer treatment that could avoid cytotoxicity of normal functional cell types

Characterization

New NP formula characterization is a vital and highly sensitive component in new drug development and delivery. Some researchers can experience poor characterization studies as a result of undefined methods or misinterpreted information [23]. The National Cancer Institute's Nanotechnology Characterization Laboratory (NCL) has taken steps in further streamlining nanoparticle formulation safety and biocompatibility. The NCL functions as a strong resource for new formulation scientist in hopes of avoiding common pitfalls in new NP design. In this review, characteristics and methods of determination will be briefly discussed as it is important to understand the varying methods used in academia and industry for identification of NP parameters. While this review offers a brief description of characterization studies commonly done in NP formulation, it is far from exhaustive. Further inquiry into proper characterization techniques should be directed towards NCL's scientific bibliography and published data on characterization [23].

1.5. Particle Size Characterization

Dynamic light scattering (DLS) is commonly used to determine NP size. DLS functions by measuring Brownian motion and relates its velocity to NP size using the Stokes-Einstein equation [24]. Reported results are expressed as mean particle size with a degree of particle homogeneity or polydispersity index (PDI). A PDI value between 0.1 and 0.25 is indicative of small distribution ranges in particle size, while PDI values greater than 0.5 suggest larger particle dispersity in relation to size [25]. Microscopy techniques can also be used to accurately determine particle size, however the process is often times more complicated, requiring several different sample preparation steps.

1.6. Determination of Zeta Potential

Measurements of particle charge, or zeta potential, are often accomplished by electrophorectic mobility assays. NPs are surrounded by two separate liquid layers, the strong inner bound Stern layer and the weak bound outer layer [25]. Electrophoresis applies an electrical current to an NP solution and measures the degree of NP mobility, thus measuring the charge of the NP outer layer. Several different pieces of equipment, such as Malvern zeta sizer and NICOMP particle sizing system, are used for particle charge analysis [26]. In many instances, these systems serve a dual functionality by providing both particle charge and particle size data analysis [27].

1.7. Measurement of Drug Release

The ability for an NP to release entrapped drug is critical in the overall functionality of NP derived drug delivery. Measurements of NP release kinetics enable scientists to properly analyze new NP system behavior and evaluate their potential efficacy in clinical use. NP release can occur through three distinct pathways: desorption of surface bound drug, polymer matrix erosion, and drug diffusion [25]. Primary routes of drug release often occur through matrix diffusion and/or erosion, while desorption of surface bound drug is commonly attributed to the rapid burst release seen in many NP formulations.

Analysis of NP release is commonly done using sample separation or membrane dialysis techniques [28]. In sample separation, NP solutions are emerged within a fixed volume of medium and placed on an electronic shaker or stir plate. Solution samples are taken at various time points and centrifuged or filtered while fresh media is added to the NP solutions. Following sample centrifugation and/or filtration, the resultant supernatant is analyzed for free drug content [27]. Dialysis methods measure NP release as a function of continuous diffusion across a membrane [25]. The method involves the use of select membrane pore size and molecular weight cut off parameters for proper NP and free drug analysis [28]. The dialysis membrane procedure consists of two compartments, a donor compartment and receptor compartment. NP solutions are placed within the donor compartment and fresh medium is placed within the receptor compartment. At various time points, samples are collected from the receptor compartment and analyzed for free drug content. Solution analysis is carried out through several different methods including high performance liquid chromatography and ultra violet spectrometry [25, 27, 28].

1.8. Morphology

NP morphological and shape characteristics are important for studying NP presence, surface characteristics, topography, degree of aggregation, and direct analysis of particle shape [29]. NP morphology studies are commonly carried out utilizing transmission electron microscopy (TEM) or scanning electron microscopy (SEM) techniques [27, 30]. In these techniques, small amounts of NP samples are placed on select TEM or SEM carbon film, with or without reflective coating [29]. Thin samples are required to allow transparency of the electron beam, as such, samples are usually coated lightly on microscopic film or if nanoparticle solutions are employed, allowed to dry before visualization [29]. TEM allows researchers to quickly and accurately achieve size distribution and particle density information for new NP formulations.

Nanoparticle Transport

A NP placed externally to cells can interact with cellular plasma membranes and enter inside the cell via passive diffusion or endocytosis [15] (Fig. 2.1). Endocytosis involves a multi stage process in which extracellular components interact with cellular membranes forming invaginations. These invaginations then become pinched to form endosomes or phagosomes (depending on route of transportation) and delivered to targeted compartments within the cell.

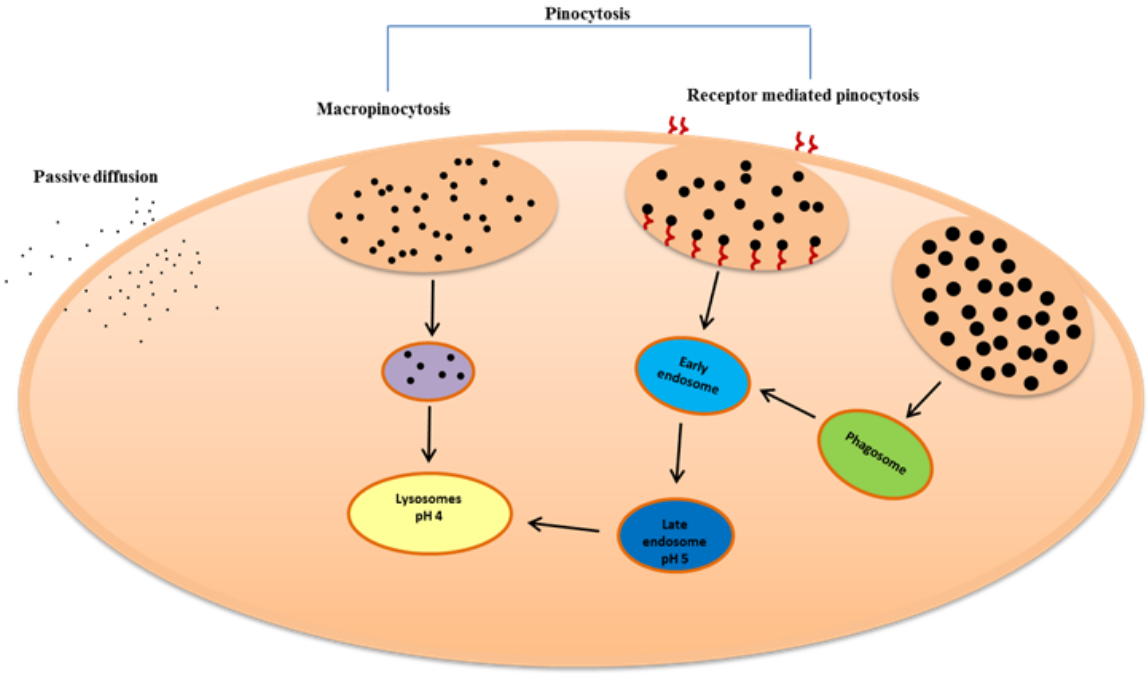

Figure 2.1 Simplified schematic representation of cellular uptake showing passive diffusion or pinocytosis and phagocytosis invagination pathways

1.9. Passive Diffusion

The process of passive diffusion can occur when a nanoparticle dissolves across the lipid membrane of a cell (Fig. 2.1). Diffusion can depend upon several factors related to nanoparticle characteristics. NP surface charge can influence the degree to which substances passively diffuse across the lipophilic plasma membrane of various cells [31]. Previous studies have demonstrated the ability of charged nanoparticles to facilitate a three to four fold increase in uptake across a cholesterol containing lipid bilayer. Further studies have elucidated diffusion of NPs within cells based on their influences on tight junctions. Sonaje *et al.* demonstrated the influence of NP formulation on the opening of tight junctions. It was shown that NP treatment resulted in slightly increased apical membrane space which facilitates paracellular transport of insulin [32]. These results indicate that the use of NPs to deliver drugs across

cellular membranes via diffusion mechanisms may offer advantages over current drug delivery strategies.

1.10. Endocytosis

The process of endocytosis is divided into two broad categories; pinocytosis (receptor mediated and/or macropinocytosis) and phagocytosis [33] (Fig. 2.1). Receptor mediated endocytosis involves the engulfment of receptors in conjunction with their associated ligand, into a coated pit. Clatherin protein coated pits are the more commonly associated receptor mediated endocytic pathway and are often referred to as the "classical" form of receptor mediated endocytosis. The clatherin coated pits are primarily responsible for uptake of essential nutrients such as cholesterol and iron [12]. Caveolae consist of structural caveolin proteins that function as integral membrane proteins and can be found in abundance in adipocytes, muscle and epithelial cells [15]. Caveolae mediated endocytosis functions by engulfing molecules that bind to caveola surfaces. Unlike clatherin dependent endocytosis, caveolae proteins do not disassociate from the vesicle following endocytosis [9]. Macropinocytosis processes allow for the engulfment of larger solute macromolecules and can measure as wide as 5 μm. Phagocytosis generally refers to cellular uptake of large microparticles such as microorganisms and dead cells, which are ingested and transported via phagosomes. It is important to note that phagocytosis is generally restricted to specialized mammalian cells, such as macrophages, that play an important role in the detection and elimination of foreign substances.

Types of Nanoparticles

Nanodelivery systems are generally divided to two classes of lipid and polymer based delivery systems.

1.11. Lipid Based Nano-Delivery Systems

Lipid based NPs have been extensively used for increased oral, topical and intravenous drug delivery [2]. Most commonly used lipid based nano-delivery systems are liposomes and small lipid based nanoparticles (Fig. 2.2A and 2.2B, respectively).

Liposomes are spherical vesicles composed of phospholipid bilayers with a particle size distribution between 10 – 1000 nm [34] (Fig. 2.2A). Liposomes are formed when phospholipids are dispersed in an aqueous solution. When phospholipids are placed in water the hydrophilic head group interacts with the polar medium resulting in the formation of multi and unilayered vesicles [35]. These vesicles are composed of biological lipid bilayers that form a spherical shell [35]. Because of their entrapment capabilities, liposomes are a highly useful tool for pharmaceutical delivery. Liposomes are normally

developed by drying down lipids from an organic solvent, then dispersing the lipids through aqueous medium in the presence of a detergent, followed by purification [36]. The most extensively used method for dispersion in liposome formation is probe or bath sonication. Characteristics of the liposome lipid bilayer can allow fusion of the liposome to cell membranes, thus instigating direct delivery of material (i.e. drug) inside the cell [37].

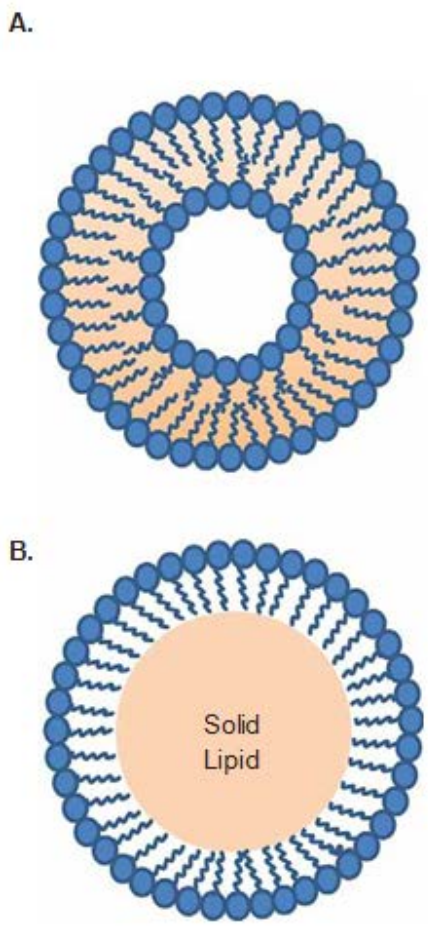

Figure 2.2(A) Structural diagram of liposomal bilayer and (B) solid lipid nanoparticle

Medical studies looking at liposomal effects of anti-cancer drugs demonstrated an increase total drug circulation and tumor exposure time brought about by increased tissue retention [38]. In fungal infections, it has been shown that amphotericin B liposomes function to enhance cellular adhesion and aid in penetrating fungal cell walls and enhance entrance into cellular cytoplasm, suggesting cell and tissue specific targeting [39]. As such, liposomes have been extensively used as carriers for many different molecules within the pharmaceutical industry. Because of their high degree of biocompatibility, low toxicity, site specificity, and ability to entrap both hydrophobic and hydrophilic compounds, liposomes have gained immense interest in commercial drug delivery [36, 40].

Solid lipid systems have become a major focus in drug delivery research. Their high degree of biocompatibility, physiochemical properties, and ability to enhance absorption of hydrophobic drugs through lymphatic uptake has made them popular compounds to utilize in drug carrier systems [40]. Compared to liquid oil solutions, solid lipids allow less formulation based drug mobility and as such, a much greater control in drug delivery [41]. Aside from effects on drug release, it should be noted that solid lipids and solid lipid nanoparticle (SLN) matrices are composed of physiological lipids which can effectively reduce acute and chronic toxicity associated with other forms of drug delivery [42] (Fig. 2.2B). SLNs are produced by mechanically dispersing lipids in water or an aqueous surfactant solution, which allows for select advantages over common liposomes and other various forms of NPs [42, 43] (Fig. 2.2B). Other advantages claimed with SLNs include: increased drug stability, high drug payload, and ease of sterilization and large scale production [42]. The enhanced stability of SLNs decreased the propensity of drug leakage and offers a delivery option that is more biodegradable and compatible and less toxic when compared to other nano-based delivery options [7, 43]. SLNs have an average particle size below 500 nm [42].

Lipid based drug conjugates are one of the most widely accepted methods for development and delivery of hydrophilic drugs. Other lipid delivery systems such as SLN experience low levels of hydrophilic drug incorporation and have low drug loading capacitance based on polarity [44]. Lipid drug conjugates (LDCs) are able to convert water soluble drugs into insoluble lipid drug conjugates by conjugation of an amino or hydroxyl group of the drug of choice to the carboxyl group present in a lipid core. LDCs are generally formed in bulk via covalent linkage or salt formation then converted into NPs via high pressure homogenization [44]. Studies have shown that the use of LDCs in drug formulation can effectively increase gut permeability and enhance oral delivery of select therapeutic agents via reduced first pass exposure and increased lymphatic absorption. Other studies have demonstrated the use of LDCs in effective increases in drug uptake across the blood brain barrier. A study conducted by Olbrich *et al.* showed that lipid conjugated diminazene demonstrated less cytotoxic effects and increased blood brain barrier passage when conjugated to a lipid matrix through enhanced LDL receptor activation [45].

1.12. Polymer Based Nanodelivery Systems

Polymer based NPs are superior to liposomes primarily because of tissue and organ specific targeting [46] (Fig. 2.3A). The ability to absorb and coat polymeric formed NPs with differing substances such as target specific ligands for tissue specificity and polyethylene glycol for increased hydrophilic properties, allows investigators and drug development specialists a wide variety in reducing toxicity and inducing specific functions for a particular drug or compound.

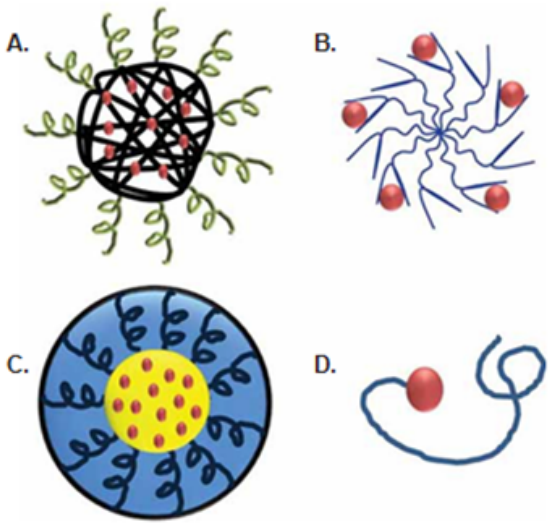

Figure 2.3 Schematic diagram demonstrating multiple polymer-based NP systems including (A) polymer-based NP with lipophilic core, (B) dendrimer-based NP with multi-valent branching arms, (C) polymeric micelles containing a hydrophilic outer shell with corresponding hydrophobic inner core and (D) drug-polymer conjugate systems

Many biodegradable polymers are available for polymer based NP formulation. Poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and poly (lactic-co-glycolic acid) (PLGA) polymers are a few of the more successfully used biodegradable polymers [46]. PLGA, PLA, and PGA are commercially available for use in various drug delivery systems. The use of PLA, PGA, and PLGA in NP formulations allows for particle manipulation without increasing toxicity as they are all biodegradable polymers that undergo hydrolysis to lactic acid and/or glycolic acid [47]. The formation of Krebs cycle intermediates associated with these polymers creates minimal physiological toxicity as they are easily and efficiently metabolized. Differing NP characteristics such as particle size, shape, and zeta potential can be produced according to parameters set forth by the specific synthesis process [48]. Degradation time for polymers can vary depending upon their molecular weight [47, 49]. Of all the biodegradable polymers currently available, PLGA seems to be the more common choice for polymer based NP synthesis and has appeared in several commercial formulations (Table 2.1) [50].

PLA is commonly used to a lesser extent due to its prolonged degradation rate, while PGA is used when fast degradation and hydrolysis processes are warranted. Particle size distributions of polymeric NPs usually range from 50 – 500 nm [51]. Unlike liposomes, polymeric NP characteristics usually employee a smaller size diameter allowing for enhanced systemic circulation and reduced recognition and degradation by the mononuclear phagocytic system [34]. Polymer based NPs have also shown an

enhanced stability and resistance to drug leakage at normal physiological conditions when compared to liposomes [52]. Inclusion of polymer formulated liposomal NP systems have been proposed and researched as a means of enhancing liposome resistance to drug leakage and prevention of potential cytotoxic effects [53]. Studies have shown that polymerization of liposomal systems can enhance liposomal resistance to drug leakage at temperatures of 40 °C.

Table 2.1
Examples of PLGA Polymers in pharmaceutical drug formulations

Polymer	Drug	Trade Name	Company	Application	Ref.
PLGA	Buserelin acetate	Suprecur depot	Sanofi-Aventis	Prostate Cancer	[151]
	Goserelin acetate	Zoladex depot	Astra Zeneca	Prostate cancer, endometrioses	[152]
	Leuprolide acetate	Eligard	Sanofi-Aventis	Prostate Cancer	[153]
	Leuprorelin acetate	Lupron depot	Takeda-Abbott	Prostate cancer, endometrioses	[151]
	Octreotide acetate	Sandostatin	Novartis Pharma	GH suppression	[151]
	Triptorelin	Decapeptyl Decapeptyl SR	Ipsen-Beaufour Ferring	Anti-cancer LHRH agonist	[151]
	Recombinant human growth hormone	Nutropin depot	Genentech-Alkermes	Growth hormone deficiency	[151]

Polymers have also been used in dendrimer delivery systems. Dendrimers (Fig. 2.3B) are highly branched molecules with multiple arms extending from a central core [54]. Aside from their highly branched structure, dendrimers also possess unique characteristics such as controlled mutlivalency, defined molecular weight and globular structure. The multivalency characteristics of dendrimers allows attachment of several different drug molecules and targeting groups to the periphery of the dendrimer. As such, dendrimers offer several advantages in drug delivery. Recently, polymeric and dendrimer science has combined to develop a new class of molecule called dendronized polymers [55]. Through polymeric conjugation, scientists can effectively alter the hydrodynamic size of dendrimers, all while maintaining particle size homogeneity and increasing drug loading capacitance [56]. Dendronized polymers have displayed favorable results in intracellular protein and drug delivery [57]. *In vitro* and *in vivo* studies have shown increased chemotherapeutic efficacy in doxorubicin (DOX) loaded dendronized polymers. Compared to I.V. delivery of free DOX, DOX loaded dendronized polymers have demonstrated a 10 fold reduction in cellular toxicity, while showing a 9 fold increase in tumor uptake

[56]. The increased anti-tumor activity of DOX loaded dendronized polymers is thought to be resultant from favorable alterations in the pharmacokinetic profile of DOX via alterations in total blood circulation time. Dendrimer based NP formulations have also been used successfully to target various viral based receptors that functions to inhibit cellular binding and entrance. Vivagel is a dendrimer based drug delivery system developed exclusively by Starpharma that contains uniquely designed polyvalent properties [20]. This dendrimeric system functions primarily through polyanionic surfaces to target various receptors. It has been shown to attach to viral surfaces and prevent binding to cell surfaces, thereby preventing cellular uptake and infection. As a result, this unique NP formulation has shown potent inhibitory effects in HIV and HSV studies while demonstrating relatively safe and tolerable effects [58, 59]. Given these results, dendronized polymers could also show favorable results in modulating the effects of various other drugs, making them ideal candidates for use in drug delivery. Polymeric micelles are formed through the self-assembly of amphiphilic macromolecules and act as efficient carriers for highly hydrophobic drugs [40] (Fig. 2.3C). The hydrophobic core of the micelle can be loaded with small hydrophobic drugs through simple emulsion or solvent evaporation techniques [60]. Their high capacity to solubilize largely lipophilic drugs make them extremely versatile tools for increasing the aqueous solubility of drugs as well as increasing and altering drug bioavailability. When factoring in the inherent and modifiable properties of polymeric micelles, they become extremely valuable commodities in the realm of drug therapeutics and delivery [61]. Polymeric micelles can be modified to control the rate of drug release, thus increasing blood circulation time and avoiding host defenses that would target drugs for degradation [62]. Relative to existing solubilizing agents such as Cremophor EL, polymeric micelles may demonstrate increased safety parameters for I.V. administration, allowing for alterations in drug dosing parameters of highly toxic and water insoluble drugs. It has been found that polymeric micelles also function by inhibiting p-glycoprotein in the gastrointestinal tract and brain, providing a way to facilitate increased drug absorption from the gut and absorption into the brain. These characteristics make polymeric micelles well suited and highly regarded for drug delivery purposes.

Like lipid based NP systems, drug conjugation utilizing polymer formulated NPs for enhanced delivery have been investigated [63] (Fig. 2.3D). Drug conjugated polymer NPs have been used in the field of research in dual purpose circumstances. A study conducted by Feng *et al.* demonstrated the use of the positively charged fluorescent polymer PFO and poly glutamic acid for the development of conjugated DOX NPs [64]. In the study, poly glutamic acid conjugates functioned to effectively delivery DOX to the site of action via endocytosis uptake, while the PFO conjugate enable successful visualization of drug/NP conjugate localization. DOX conjugated polymer NPs have also been shown to facilitate tumor cell localization and controlled drug release via enzymatic and pH sensitive methods. A

recent study showed polymer conjugates of DOX bound via pH sensitive hydrazone or enzymatically degradable amide bonds facilitated differing anti-tumor mechanistic actions [65]. The mechanism of differing actions was thought to be a result of amide linkage formulations tendency to directly penetrate the plasma membrane leading to accumulation of DOX throughout subcellular compartments [65, 66]. Conjugate NPs formulated with pH sensitive hydrazone bonds demonstrated cellular uptake via endocytosis and pinocytosis mechanism, leading to DOX release in low pH endosomes and lysosomes [65]. As such, it is believed that the differing mechanisms of action for both conjugate formulations could potentially lead to synergistic anti-tumor effects with low drug specific toxicity [67].

1.13. Metal Based Systems

Metal based NPs have been commonly seen in industry and academia. Of particular interest has been the utilization of gold based NPs for cancer treatment. The characteristic shine that is common with gold NPs and the ease of localization and visibility makes them a great tool for future research, both diagnostically and preventatively, in cancer therapy and treatment [68]. The dynamic in binding affinity of gold NPs to non-cancerous cells versus cancer cells makes them excellent choices for cancer identification as gold based NPs have a 600 percent greater affinity for cancer cells than for noncancerous cells. The abundance of cancer cells that exhibit epidermal growth factor receptor (EGFR) makes it possible to conjugate or bind an antibody for EGFR to gold NPs, thus allowing them to attach to their targeted cancer cells [69]. Various gold based nanoparticles are being used in preclinical and *in vitro* studies for the delivery of other chemotherapeutic compounds as well [70]. Drugs such as 5-fluorouricil and docletaxel have been reformulated into gold nanoparticle delivery systems for the treatment of colon and squamous cancer lines. A recent study demonstrated the use of gold NPs conjugated with partially polymerized liposomes for DOX delivery. The study showed an enhanced killing effect of MDA-MB-231 breast cancer cell lines upon controlled release of DOX loaded polymeric-gold NPs [53]. Gold NPs have also been used for the induction of hypothermic injury in various cancer cell lines and have been shown to be effective in the treatment of superficial tumors. As such, clinical trials using hypothermic techniques coupled with gold NPs are currently under way for patients suffering from oropharyngeal malignancies.

Platinum NPs are easily synthesized through the reduction of hexachloroplatinate with hydrogen gas [71]. One of the most surprising characteristics of platinum NPs involves their antioxidant properties [72]. Platinum NPs have been found to exude antioxidant properties and prolong the life span of *C. elegans* during experimental analysis. Recently, platinum based NPs have demonstrated increased efficacy of chemotherapeutic agents in the treatment of a variety of breast and ovarian cancer cell lines [73].

Platinum nanoparticles were shown to reduce toxicity profiles commonly associated with chemotherapeutic drug use and significantly increase drug anti-tumor efficacy. These findings suggest that the beneficial properties of platinum NPs may extend beyond their antioxidant properties and offer novel advantages in drug delivery.

The magnetic properties and biocompatibility of superparamagnetic iron oxide nanoparticles (SPION) has facilitated their emergence into the field of biomedicine as a promising agent in medical therapeutics and diagnostic. The high magnetization properties of SPIONs allows for their use as excellent image probes for MRI contrast and imaging. It has been found that SPION based contrast agents induced longer delineation of tumor margins and enhanced tumor localization in comparison to conventional contrast agents such as gandolinium [74]. As such, FDA approval has been given to several iron oxide based imagining agents such as Lumiren and Endorem for use in the medical field [74, 75]. Iron oxide has also found successful use in the field of disease treatment [74]. Drug loaded iron oxide NPs have shown enhanced caspase-8 activity in human cancer cell lines with increased permeation of cellular membranes brought about by tight junction disruption [76]. Other studies have shown further cellular permeability induced by iron oxide based nanoparticles as well as enhanced tumor cell targeting via surface bound peptide interactions, making iron oxide based NP formulation an important potential aid in disease treatment [74].

1.14. Carbon Nanotubes

Carbon nanotubes (CNTs) consist of a hexagonal arrangement of carbon atoms consisting of one or more walls of graphene sheets [77]. The highly toxic nature of first generation CNTs made them unsuitable for application in a variety of fields such as drug delivery. However, recent advances in CNTs formulation has enable researchers to significantly reduce the toxicity profiles commonly associate with CNT delivery [78]. Through surface modification and changes in covalent and non-covelent intermolecular interactions, researchers may be able to synthesize altered CNTs with reduced toxicities profiles. Given the capabilities of reducing toxic side effects, research into drug delivery using CNT has risen in popularity over the past few years. CNT has shown improvements for the delivery of a variety of different molecules [77]. Mehra et al. used multi-walled CNTs in the reformulation of DOX for cancer treatment. In the study it was found that drug formulations utilizing CNTs presented with increased total circulation time, sustained release and enhanced DOX cytotoxicity when compared to standard DOX dosages [78]. Sustained release of other molecules has also been reported [79]. CNTs are thought to facilitate cellular uptake by translocating across cellular membranes through either endocytosis or non-endocytosis pathways [77]. CNTs have demonstrated the ability to enhance permeability and extravasate

in tumor tissue. When coupled with their large surface area and subsequent high loading capacity, these properties make them ideal candidates for new approaches to drug delivery.

1.15. Quantum Dots

Quantum dots (QDs) are described as basic semiconductors that exude electrical characteristics with a particle size distribution between 2 – 10 nm [80, 81]. These specific dots are developed and manufactured through various techniques such as fabrication, viral assembly of inorganic nanocrystal, and electrochemical assembly involving ionic reactions between electrolyte and metal structures [80]. QDs contain a larger number of sites in which active material can be attached. As a result of the large number of active sites contained on QDs, multifunctional properties can be developed through tethering and modification of an assortment of bioactive agents [82]. Recent studies have shown that pegylated QDs enhanced cellular uptake and increased NP trafficking through targeting of the caveolae mediated endocytic pathway [15]. Other studies have demonstrated the use of QDs in tissue specific targeting of tumor cells by tethering prostate specific antigen to QDs [82]. Based on the increasing versatility of quantum dots, their use in comparison to less versatile organic dyes are rising exponentially [80].

1.16. Protein Based Systems

Protein based NP systems have been developed to aid the delivery of certain drugs to specific, localized compartments [83]. Abraxane is an albumin bound NP containing the chemotherapeutic agent paclitaxel which was approved by the FDA for use in relapsed and metastatic breast cancer [15]. Abraxane functions by taking advantage of receptor mediated endocytosis of tumor cells [84]. It selectively binds to GP-60 albumin receptors located within cell caveolae. This binding facilitates movement into the interstitial space where it is engulfed by secretory tumor proteins. In turn, these tumor proteins are taken up by tumor cells resulting in cell specific tumor cytotoxicity [85].

Nanoparticle Preparation

Various means of preparation, solvent evaporation, high pressure homogenization, nanoprecipitation, salting out, microemulsion, and detergent removal have been identified for NPs. As the field grows at a substantial rate, a wide variety of preparation methods have been developed for nano-material formulation. However, there are several key properties of NPs and drug encapsulation that one must keep in mind. The selection of material to be used for formulation depends on several factors involving,

but not limited to, the nanometric size of particles needed, surface characteristics required, properties of the drug one wishes to encapsulated and the bioavailability and toxicity limits required [46]. In this article, we discuss only solvent evaporation and high pressure homogenization.

1.17. Solvent Evaporation

Solvent evaporation techniques are the most commonly used techniques for polymer based NP preparation [51]. Solvent evaporation is most prevalently used in biotech and pharmaceutical industries and represents the most common technique seen in the literature. There are two types of procedures typically used when preparing NPs through solvent evaporation, single emulsion and double emulsion [46]. Single emulsion preparation involves oil in water emulsions, while double emulsion techniques utilize water in oil in water emulsions [46]. Once solvents are properly prepared, they are homogenized and evaporated to facilitate the formation of solid NPs. Solid NPs are collected by ultracentrifugation then washed with distilled water to remove additional surfactants. After additional surfactants are removed the mixture is lyophilized.

The step by step solvent evaporation process is as follows: Polymers are dissolved in organic solvents, followed by addition of the drug of choice [46]. The organic mixture is added into a water based phase containing an emulsifier or stabilizer (oil/water). The mixture is then homogenized or sonicated for complete homogenization, after which the solution is then stirred constantly for several minutes to evaporate organic phase and harden newly formed NPs. Finally, solutions undergo centrifugation or filtration to harness newly formed drug particles (Fig. 2.4).

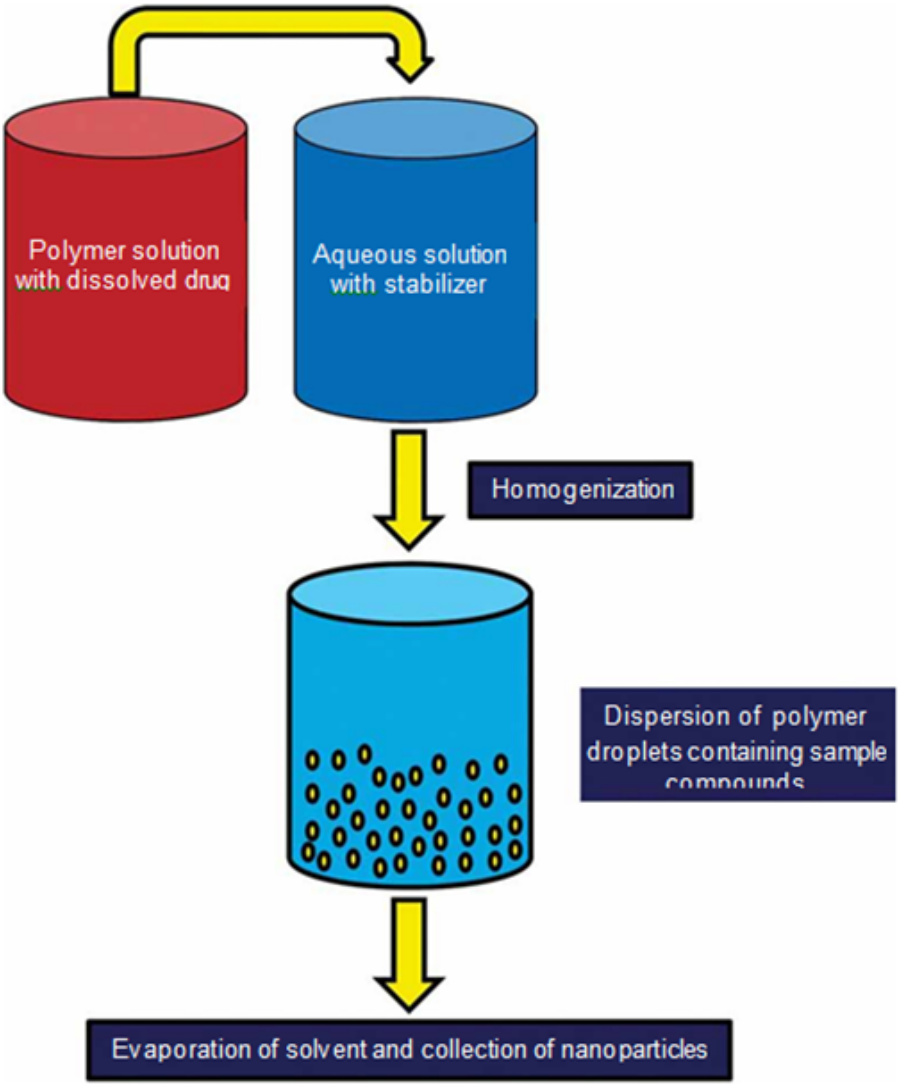

Figure 2.4 Schematic representation of solvent evaporation processes commonly used in nanoparticle production showing separate organic and aqueous phase solution with dissolved constituents and formation of particle dispersion.

1.18. High Pressure Homogenization

High pressure homogenization (HPR) is a technique commonly used in the preparations of SLNs and liposomes [41]. HPR can be performed as either a hot homogenization or a cold homogenization (Fig. 2.3). For hot HPR techniques, a drug is usually dissolved in lipid being melted at 5-10°C above its

melting point. The melt is then dispersed under a hot aqueous surfactant solution that is being heated at the same temperature. The resulting solution is then homogenized to form a hot oil in water nanoemulsion and cooled to room temperature to enable recrystallization and the formation of lipid vesicles (Fig. 2.5A). In cold HPR, the drug containing melt is cooled to form a solid lipid. The solid lipid is then ground to form microlipid particles. Microlipids are then dispersed in a cold aqueous surfactant solution and homogenized at or below room temperature to facilitate SNL formation (Fig. 2.5B).

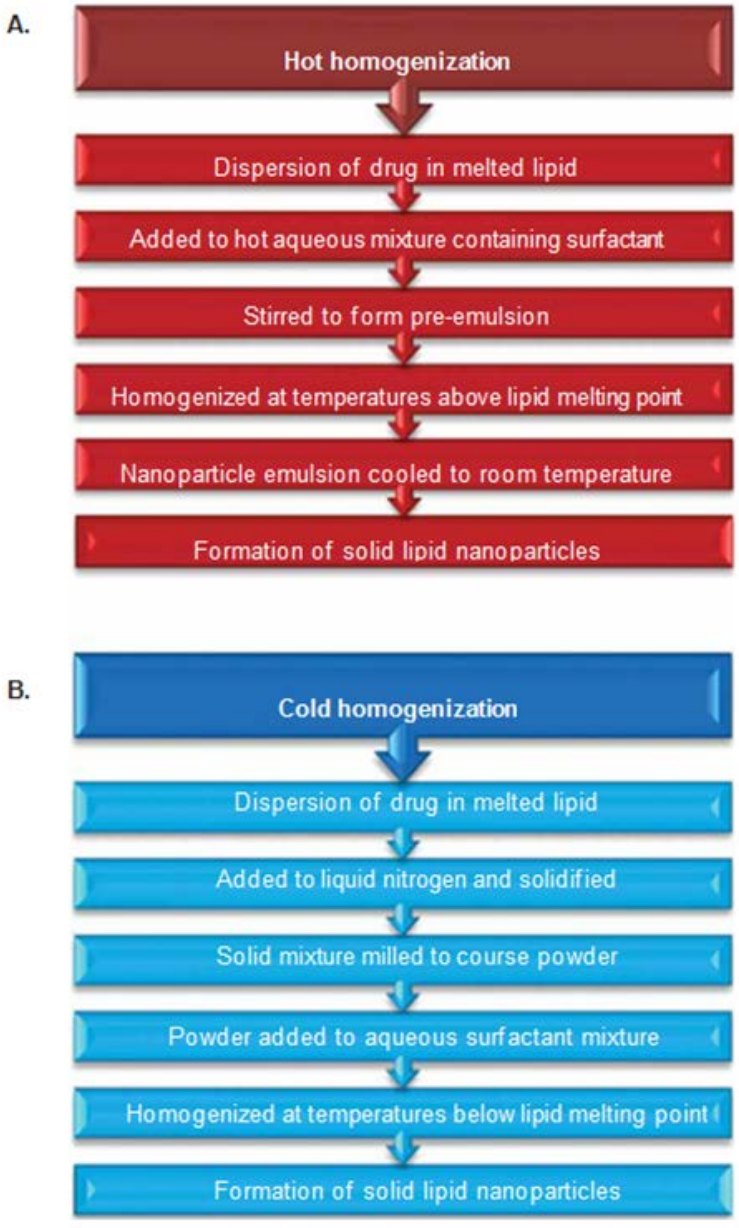

Figure 2.5 Diagram showing the technique for making SLNs by (A) hot homogenization and (B) cold homogenization.

1.19. Biodegradation

A key aspect in NP abilities to control and alter pharmacokinetic and pharmacodynamic parameters is biodegradation. NP biodegradation is thought to occur primarily through hydrolytic mechanism that can be enhanced or reduced dependent upon various factors. For polymer based NPs, molecule weight (MW), ratio, and composition play a key role in determining the extent or rate or degradation. A study conducted by Kamei *et al.* utilized two differing weights of PLGA (10,000 and 20,000 MW) in determinations of altered degradation rates [86]. It was found that the low molecular weight polymer exhibited a rate of degradation of 8 days, while the degradation of high MW polymer was doubled to that of 16 days. It is thought that molecular weight ranges can play a role in polymer degradation as well [87]. Compounds with a large MW range present with higher levels of carboxylic end groups that could function to increase catalytic degradation. As such, polymers of a wider MW range would be expected to facilitate or increase degradation whereas polymers with smaller more compact MW ranges would present with less carboxylic end groups thus reducing catalytic activity and degradation.

Composition also plays an important role in overall release kinetics. A study conducted by Tabata *et al.* examined the biodegradation of particles composed of lactic acid polymer, glycolic acid polymer, or copolymers consisting of differing ratios of lactic and glycolic acid [88]. It was found that the rate of particle degradation was controlled by altering monomer composition [87]. Particles containing 50% glycolic acid and 50% lactic acid elicited the fasted rate of degradation, while particles composed of higher or lower concentrations of glycolic acid were found to degrade at a slower rate. As such, drugs entrapped within a polymer matrix can elicit sustained release profiles through slow diffusion from the polymer core and degradation of the polymer matrix [12, 87]. The rate of degradation of NPs can vary dependent upon several factors allowing for the design of various NP rates of drug release based on polymeric MW ranges or core polymer composition [89].

1.20. Bioavailability Improvement

Oral solid drug delivery systems are a common and convenient form of drug administration. For an increasing numbers of drug molecules, the oral route is complicated by solubility, bioavailability, and, stability concerns that necessitate higher oral doses or parenteral administration [90]. These considerations are particularly applicable to Biopharmaceutics Classification System (BCS) Class II drugs that exhibit poor solubility and high permeability [91]. For a lot of drugs, the use of NPs is meant to improve oral bioavailability by decreasing particle size and increasing relative surface area to facilitate drug dissolution. A recent study looked at oral absorption and the effects of high pressure homogenized

nanosuspensions of itraconazole. It was found that drug nanoization resulted in a faster rate of dissolution accompanied by an increase in total blood plasma concentrations [92]. A recent study looked at the inclusion of orally dosed GLP-1 agonist in nanomatrices composed of Eudragit and silica [93]. The highly sensitive nature of GLP-1 to acidic conditions of the stomach makes it a relatively poor candidate for oral administration. However, when GLP-1 was adsorbed on the surface of solid silicon based nanoparticles and encapsulated with the pH-sensitive polymer, Eudragit, it displayed a five-fold higher degree of mucosal adhesion and proteolytic stability within the gastrointestinal tract.

1.21. Pharmacokinetics Alterations

Characterization of drug NP *in vivo* kinetics is further complicated by the unique attributes of each drug, excipient, and route of administration. A simplification of available reports is the use of NPs increases the area under the curve (AUC), a common measure of total drug exposure. Improvements in bioavailability relative to current preparations helps explain these observations [94]. It is important to note that as bioavailability increases, access to various body compartments increase as well [95]. Changes in distribution will profoundly impact other pharmacokinetic parameters, including drug clearance (Cl), volume of distribution (Vd), maximum drug plasma concentration (Cmax), time of maximum drug plasma concentration (Tmax), elimination rate constants, and drug half-life. Drug concentration-time profiles for NPs can reflect either immediate or extended release characteristics as a function of baseline solubility when compared to commercially available preparations. Drugs with poor solubility, like BCS Class II compounds, will have their solubility enhanced by nanoparticles and achieve a higher Cmax in less time (lower Tmax) [91]. The converse is true of more hydrophilic compounds, where NPs will delay a compound's dissolution [96]. Other extended release applications use pH-dependent matrices to help conserve stability throughout the gastrointestinal tract [4].

1.22. Side Effect Reduction

Traditional examples of improved side effect profiles include amphotericin and cyclosporine [97]. More recent studies have addressed the severe toxicity associated with chemotherapeutic agents. Cremophor EL is a toxic solubilizing agent used in the formulation of highly lipophilic drugs, such as paclitaxel [98]. Recent studies comparing new nanometric formulations of paclitaxel with Cremophor based vehicle formulations have shown favorable results in drug response, safety and side effect profiles. Paclitaxel nanosuspension has also been shown to exhibit distinct distribution profiles that may complement their tolerability in the foundation of new applications in liver, lung, or spleen metastases [95]. NP renal side

effects in regards to chemotherapeutic and antibiotic reformulation are further expounded on within this review.

7.4.1. Antibiotic Drug Induced Nephrotoxicity

Amphotericin B is a popular anti-fungal agent that is considered the gold standard for the treatment of an assortment of fungal based diseases [99]. However, amphotericin B use has been linked to a variety of adverse side effects, the most common being nephrotoxicity [100]. Nephrotoxic effects of amphotericin B are very high and can produce acute renal failure symptoms in patients [101]. Previous studies have shown that 49 - 64 % of patients administered amphotericin B developed acute renal failure symptoms [102]. When compared to baseline, 29 % of patients show serum creatinine concentrations greater than 250 mmol/L [103].

Due to the extreme toxic nature of amphotericin B, reformulation tactics have been used to minimize toxic side effects. Studies have demonstrated that the use of biodegradable NPs significantly reduce nephrotoxic side effects of amphotericin B while still maintaining potent anti-fungal activity [104]. Several studies have looked at specific renal cell effects of amphotericin B formulated NPs. In one study, lactate dehydrogenase release was measured as an indicator of cell damage. It was shown that amphotericin B formulated NPs reduced cellular lactate dehydrogenase release, indicating reduce nephrotoxicity [105]. Another study showed reduced protein expression in cell lines treated with base amphotericin B in comparison to NP formulated amphotericin B, indicating mechanistic actions on protein synthesis as a possible means of NP reduced nephrotoxicity [105, 106].

Vancomycin is a potent antibiotic drug used in the treatment of multi-drug resistant bacterial infection [107]. Although its high efficacy in regards to treatment of infection is ideal, it has been shown to increase serum creatinine concentrations and cause severe nephrotoxic side effects [108]. Poly ethylene glycol is an excipient commonly used to stabilize and solubilize pharmaceutical products, when low molecular weight variants are wanted. Recent studies have shown that solubilized formulations of vancomycin utilizing D-mannitol and poly ethylene glycol effectively reduced renal side effects of vancomycin when administered at the nephrotoxic dose of 400 mg/kg in rats [109]. Groups receiving reformulated vancomycin showed no marked changes in BUN parameters and experienced no alterations in histological parameters of the kidneys when compared to control. At twice the daily clinical dose, renal tissue accumulation of vancomycin increased substantially in groups receiving conventional formula (653.8 ± 186.0 µg/g) when compared to groups receiving vancomycin reformulation (442.9 ± 120.6 µg/g) [110]. These findings suggest that vancomycin reformulation reduces

renal damage and prevents nephrotoxic side effects through modifications of drug release and tissue targeting.

7.4.2. Chemotherapeutic and Immunosuppressive Drug Induced Nephrotoxicity

Cyclosporine (CsA) is a potent chemotherapeutic and immunosuppressing agent used for the prevention of graft and organ transplant rejection and the treatment of certain cancers and autoimmune disorders [111]. CsA is a highly toxic drug that may induce sever nephrotoxic symptoms ranging from acute kidney failure to chronic renal disease [112]. As such, it is important to develop a delivery system that could serve to alter drug efflux, tissue accumulation, and system circulation for avoidance of adverse effects [104, 113]. Several studies have reported the benefits of polymeric micelle cyclosporine encapsulation on pharmacokinetic parameters as well as drug induced nephrotoxicity symptoms [114, 115]. In a study conducted in 2005, it was found that volume of distribution and clearance values of micelle encapsulated cyclosporine was 10 and 7.6 fold lower than conventional formulas, while tissue accumulation of cyclosporine in the kidneys experienced a 1.4 fold reduction in comparison to groups receiving standard drug delivery [114].

Tacrolimus is a potent immunosuppressive drug. [116]. Originally marketed for the prevention of organ transplant rejection, tacrolimus has recently gained wide spread attention for its use in the treatment of inflamed bowel disease (IBD) [117]. Recent studies discovered that the immunosuppressive effect of tacrolimus was not selective to inflamed tissue [117]. As a consequence of tacrolimus non-selectivity, it was found to cause severe nephrotoxic symptoms in patient receiving drug treatment for IBD. Interestingly, the effectiveness of NP formulations on reducing nephrotoxic side effects commonly associated with tacrolimus use has been reported. NP reformulation of tacrolimus has been successfully used for the development of tissue specific drug delivery and enhancement in phagocytosis mediated cellular uptake [118]. It has been shown that PLGA formulated tacrolimus NPs can effectively target lymphatic T cells for enhanced immunosuppressive properties [119]. A recent study looked at both PLGA and Eudragit formulated tacrolimus nephrotoxicity and found that drugs treated with NP formulated tacrolimus exhibited similar creatinine and BUN levels as compared to untreated controls [117].

Cisplatin is a highly effective chemotherapeutic agent used in the treatment of various cancers [120]. The most common side effect associated with cisplatin use is nephrotoxicity of the proximal tubule [121]. As such, altered formulations of cisplatin for patients receiving chemotherapy treatments are highly desirable to prolong quality of life and drug effectiveness [122]. Several studies have been performed on the use of NPs and cisplatin encapsulation with promising results. Cisplatin incorporated polymeric

micelles have been shown to reduce or eliminate nephrotoxic side effects while prolonging its anti-tumor activity [123]. Rats receiving cisplatin polymeric micelle formulations showed no marked changes in nephrotoxic parameters such as BUN, creatinine, or tubular damage and necrosis when compared to rats receiving control [124]. Cisplatin is thought to effect renal toxicity through up regulation of reactive oxygen species (ROS) and increases in oxidative stress [125]. Recent studies have shown the ability of NPs to increase effectiveness of ROS scavengers during co-administration with cisplatin. ROS scavengers such as selenium have been shown to prevent or reverse cisplatin induced nephrotoxicity above control when co-administered as NP encapsulated product [126]. Cisplatin is highly reabsorbed in the kidneys [121, 127]. The degree of drug reabsorption is thought to play a role in the onset of nephrotoxic side effects associated with cisplatin use [127]. It is thought that liposomal formulated cisplatin functions to reduce or diminish tubular reabsorption of cisplatin resulting in a reduction in nephrotoxic side effects. A recent clinical human study looked at the benefit of dual combination of paclitaxel and liposomal formulated cisplatin in comparison to traditional formulated cisplatin with paclitaxel [127]. The study showed a marked reduction in toxic side effects, including nephrotoxic effects, with adverse side effects mainly occurring in patient groups receiving traditional formulation. A statistically significant decrease in nephrotoxic side effects was noted in groups receiving liposomal reformulated cisplatin when compared to traditional cisplatin formulation ($P < 0.001$).

Another chemotherapeutic agent, DOX, is one of the most important drugs used for the treatment of malignancies and tumors [128]. It has been shown that NP formulated DOX effectively enhances systemic circulation time, increases cellular drug uptake via macropinocytosis, and possibly down regulates P-gp efflux [129]. Formulation alterations involving the use of lipomers and NPs have demonstrated favorable outcomes in regards to increased bioavailability, decreased toxicity, and increase in kidney tissue specific antioxidant capacitance of doxorubicin [130-132]. Similar to co-administration of cisplatin and selenium, these studies show elevated antioxidant parameters along with reduced levels of lipid peroxidation in renal tissue samples of rats receiving orally formulated NP-Dox compared to conventional formula [130]. These findings suggest that NPs may play a role in both direct and indirect means of renal adverse effect prevention.

7.4.3. Potential Use of Nanoparticle Formulations in Decreasing Renal Side Effects of Nonsteroidal Anti-Inflammatory Drugs

Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), has been successfully formulated for ocular, transdermal and colonic delivery using NPs with promising results [133-135]. Oral formulations of diclofenac have been developed using polymer based NPs that altered pharmacokinetic parameters

resulting in lower systemic exposure and faster absorption in comparison to standard diclofenac formulations [4]. A recent phase 2 study using diclofenac loaded NPs showed increased tolerability and enhanced drug efficacy and pain relief in patients suffering from acute dental pain [136]. Diclofenac loaded SLNs have also demonstrated enhanced systemic circulation and increased permeation in ocular delivery [137]. Indomethacin, another non-selective NSAID, has shown results in NP formulations as well. In recent experiments, NP encapsulated indomethacin has demonstrated increased bioavailability, prolonged drug release, and a higher degree of anti-inflammatory activity [138]. Recent NP conjugates utilizing indomethacin have shown enhanced cellular uptake compared to base indomethacin. In a study conducted by Li *et al.* indomethacin was successfully conjugated to heparin, which resulted in increased indomethacin uptake into human nasopharyngeal carcinoma cell lines via increased drug permeability and endocytosis pathway uptake [139].

Celecoxib is a highly metabolized cyclooxygenase (COX)-2-selective inhibitor known to increase risk of cardiovascular and renal side effects [140, 141]. As a result of its high rate of metabolism, higher dosages of celecoxib are often required to reach effective concentrations at the site of action [142]. Several studies involving celecoxib entrapped NPs have been performed that elucidate their effectiveness at overcoming celecoxib bioavailability issues [91, 143]. A study conducted by Morgen *et al.* showed that orally administered NP loaded celecoxib resulted in an increase in bioavailability and faster time to peak plasma concentrations [91]. Another study showed that intra-articular injections of celecoxib formulated small lipid NPs enhanced site of action and drug retention in patients suffering from joint pain [144]. Additionally, two other studies demonstrated increased anti-inflammatory properties and tumor growth inhibition of celecoxib entrapped NP formulations during subcutaneous delivery [145]. Celecoxib loaded NPs have shown the ability to undergo strong binding and cellular uptake via non-endocytosis pathways in HT-29 cancer cells [146]. Cytoplasmic and nuclei localization was also observed in HT-29 cell lines as a consequence of non-specific interactions between the charged surfaces of NPs and the negatively charged cellular membrane.

NP formulated NSAIDs offer a wide variety of benefits in terms of altered pharmacokinetic parameters. Their impact on NSAID related gastrointestinal side effects are well documented. It has been shown that NP formulated NSAIDs decrease incidences of gastric irritation and ulceration in comparison to conventional delivery systems [147]. Conversely, the use of NP formulations in NSAID therapy for carcinoma treatment has shown favorable results. Recent studies show the use of celecoxib loaded NPs resulted in 78% to 95% reduction in cell proliferation with a marked increase in apoptosis in colon cancer cell lines [146]. Interestingly, even though renal side effects are some of the more adverse

occurrences associated with NSAIDs, the beneficial effects of NP formulated NSAIDs on renal and cardiorenal side effects are yet to be elucidated.

Conclusion

Nanoparticle formulation presents many advantages for drug delivery. Depending upon individual needs and interest, scientist can easily modify NP formulations for a variety of different compounds. The ease of use in developing NPs makes them ideal for utilization within multiple areas of research. NPs offer a plethora of biological advantages. They can protect drugs from damage and oxidation, act as a drug stabilizer, increase tissue specific targeting, and aid in diffusion. Variations in surface modifications for NPs allow flexibility in drug design and optional modifications based on individual research needs. NP's can improve the pharmacokinetic and pharmacodynamics profiles of a variety of drugs and can reduce drug toxicity and increase drug safety parameters. NP formulations offer a highly feasible alternative in the realm of new drug development. They have been shown to effectively work in reducing major side effects in many applicable drugs of today. As such, the use of NPs in new drug formulation offers a promising alternative to side effect, risk reduction and overall patient safety and quality of life.

Expert Opinion

NP use in drug delivery is increasing exponentially as the elucidation of their effectiveness progresses in the field of medicine and bioscience. The use of NPs offers the medical researcher a plethora of alternative avenues in the investigation and elucidation of effects in drug delivery on clinical efficacy and outcomes. As such, researchers are investigating the use of NPs in a range of medical avenues from image enhancement to skin graft procedures [148]. The potential for NP based drug delivery is virtually limitless and is measurable only by our current understanding of mechanistic and molecular properties that facilitate NP function. In the future, NP drug delivery should find inclusion into a number of prescription based medical products above and beyond those currently seen in the literature and the medical community today. At present, the pharmaceutical industry is investigating altered drug delivery methods for new and currently patented drugs as a means to offset exponential cost in novel new drug development and prevention of attrition [149]. Changes in formulation design and drug delivery have been found to effectively increase the efficacy and therapeutic use of various drugs. The current understanding of NP based drug delivery designs lends itself to industrial utilization to spur economic growth and/or offset economic loss brought forth by new drug development. These altered delivery methods can provide companies with avenues to change overall effectiveness and functionality of

current popular medicines used in multiple disease based therapies. As such, the use of NP technology should experience significant growth in industrial sectors not only for their ability to increase current drug effectiveness but also for their ability to facilitate new targeted delivery and stimulated economic growth.

Table 2.2
Examples of FDA approved drugs employing nanotechnology

Trade Name	NP Technology	Company	Route	Therapeutic Class	Ref.
Genexol-PM (paclitaxel)	Polymer Micelle	Celgene	IV	Chemotherapeutic	[154]
DaunoXome (daunorubicin)	Liposome	Galen	IV	Chemotherapeutic	[155]
Doxil (doxorubicin)	Liposome	Janssen	IV	Chemotherapeutic	[156]
Neoral (cyclosporine A)	Microemulsion	Novartis Pharma	Oral	Immunosuppressive	[157]
Rapamune (sirolimus)	Ball Milling	Wyeth	Oral	Immunosuppressive	[158]
Tricor (fenofibrate)	Ball Milling	Abbott	Oral	Antihyperlipidemic	[158]
Triglide (fenofibrate)	High Pressure Homogenizatio n	Shionogi	Oral	Antihyperlipidemic	[158]
Emend (aprepitant)	Ball Milling	Merck	Oral	Antiemetic	[159]
Megace ES (megestrol)	Ball Milling	Par Pharmaceutical	Oral	Appetite stimulant	[158]
AmBisome (amphotericin B)	Liposome	Gilead	IV	Antifungal	[160]
Abelcet (amphotericin B)	Liposome	Sigma-Tau	Injection	Antifungal	[161]
Caelyx (Doxorubicin)	Liposome	Janssen	IV	Chemotherapeutic	[161]
Vivagel (SPL7013)	Dendrimer	Starpharma	Vaginal	Antimicrobial	[20]
Depocyt (Cytarebine)	Liposome	Sigma-Tau	Injection	Chemotherapeutic	[162]

Although seldom addressed in such reviews, it is important to note that production cost and model scale-up for reformulated NP designs would present itself as a major hurdle in the continuation of any NP drug creation. While the examples illustrated within the context of this article are promising in regards to the use of NPs as novel treatment alternatives for the future, several formulations have not

processed beyond pre-clinical studies based on large scale production cost and NP formulation reproducibility. Fortunately, the advent of new and emerging FDA regulated NP based drugs has led to an increase in industrial capabilities in NP production (Table 2.2).

Currently marketed NP drug designs have paved the way for easier transition of model scale up for a variety of new NP drug products that may come in the future. Unfortunately, not all NP systems have been recognized in a mass market approach, leaving avenues of scale up and mass production susceptible to select NP systems.

Further research into differing avenues of NP delivery must be highlighted for a complete understanding of most effective routes of administration. At present, studies done with NP reformulated drugs have focused primarily on oral, intravenous, or transdermal delivery. Research into the effective avenues of NP formulated drug administration must be taken into account in order to properly understand the pinnacle effects a new drug delivery system utilizing NPs can offer. NPs can alter various properties of a drug, which in turn affects the traditional method of drug delivery. It is important to highlight differing modes of administration to properly gauge effects of NP drug reformulation on delivery. For example, the use of PLGA is known to impart hydrophobic properties of NP encapsulated drugs based on the extent of lactic acid content [150]. The alteration in drug lipophilic properties can theoretically act to alter a drugs best means of delivery. As such, dependent upon the investigators chosen NP system for delivery, emphasis should be placed on not only the effects of NP encapsulated drug delivery but the effect of NP encapsulated drugs in various delivery methods. Further clarification in best fits for modes of delivery will further strengthen use of NPs in newly developed pharmaceutical products.

In this article, we have highlighted many examples of drug induced nephrotoxicity, summarized in Table 2.3. The beneficial aspects of NP formulated alternatives in renal complications seen within this review supports the possibility of creating viable drug alternatives in hopes of quenching and/or reducing major treatment side effects. Unfortunately, further studies are still needed to clarify the extent of their benefits and the overall best practice in terms of NP design. Exact mechanistic approaches for the reduction of renal side effects noticed with NP entrapped drugs are not completely understood at the present time. Further investigation into the mechanistic effect NP design has on drug excretion, kidney accumulation, and cellular uptake is warranted to fully understand pharmacodynamics and pharmacokinetic effects in relation to nephrotoxicity and onset of renal side effects. Also, renal complications in relations to NSAID consumption are commonplace for a variety of COX inhibitors. Surprisingly, few, if any studies have been performed looking at known COX inhibiting NP

formulations and their effects on renal function. Further studies to elucidate the effectiveness of NP formulations on NSAID induced nephrotoxicity are highly warranted.

Declaration of Interest

The authors state no conflicts of interest regarding the opinions expressed and have received no payment in preparation of this manuscript.

Table 2.3
Example of NP effects on drug induced nephrotoxic side effects

Drug Name	Category	Treatment Indications	NP Formulation Effects on Nephrotoxicity	Ref.
Amphotericin B*	Antibiotic	Fungal infections	Reduced nephrotoxic parameters in human clinical trials, decreased tubular cell damage, reduce creatinine clearance	[104]
Vancomycin	Antibiotic	Bacterial infections	Reduced renal damage and kidney drug distribution	[110]
Cyclosporine*	Chemotherapeutic	Cancer, autoimmune disorder, organ transplant rejection	Reduced kidney drug accumulation, reduced total BUN and plasma creatinine, prevention of glomerular damage, improved drug bioavailability	[113]
Tacrolimus	Immunosuppressive	Inflamed bowel disease, organ transplant rejection	Reduced creatinine and BUN levels, maintenance of normal kidney function compared to non-treated controls	[117]
Cisplatin	Chemotherapeutic	Various cancers	Prolonged anti-tumor effects, reduced nephrotoxic side effects in human clinical trials, reduce drug accumulation within kidneys, reduction in kidney cell death	[73]
Doxorubicin*	Chemotherapeutic	Various cancers, tumor therapy	Enhanced drug bioavailability, increased antioxidant parameters in kidney tissue, reduced renal tissue based lipid peroxidation	[163]

*NP formulations with FDA approval and market availability

Figure Legends

Figure 2.1. Simplified schematic representation of cellular uptake showing passive diffusion or pinocytosis and phagocytosis invagination pathways.

Figure 2.2. Structural diagram of liposomal bilayer (A) and small lipid nanoparticle (B).

Figure 2.3. Schematic diagram demonstrating multiple polymer based NP systems including (A) polymer based NP with lipophilic core, (B) dendrimer based NP with multivalent branching arms, (C) polymeric micelles containing a hydrophilic outer shell with corresponding hydrophobic inner core, (D) and drug-polymer conjugate systems.

Figure 2.4. Schematic representation of solvent evaporation processes commonly used in NP production showing separate organic and aqueous phase solution with dissolved constituents, and formation of particle dispersion.

Figure 2.5. Diagram showing the process of (A) hot homogenization and (B) cold homogenization techniques.

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CHAPTER 3

Design and Optimization of PLGA-Based Diclofenac Loaded Nanoparticles

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Keywords: NSAIDs; Nanoparticles; Formulation; PLGA; Diclofenac

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Abstract

Drug based nanoparticle (NP) formulations have gained considerable attention over the past decade for their use in various drug formulations. NPs have been shown to increase bioavailability, decrease side effects of highly toxic drugs, and prolong drug release. Nonsteroidal anti-inflammatory drugs such as diclofenac block cyclooxygenase expression and reduce prostaglandin synthesis, which can lead to several side effects such as gastrointestinal bleeding and renal insufficiency. The aim of this study was to formulate and characterize diclofenac entrapped poly(lactide-co-glycolide) (PLGA) based nanoparticles. Nanoparticles were formulated using an emulsion-diffusion-evaporation technique with varying concentrations of poly vinyl alcohol (PVA) (0.1, 0.25, 0.5, or 1%) or didodecyldimethylammonium bromide (DMAB) (0.1, 0.25, 0.5, 0.75, or 1%) stabilizers centrifuged at 8,800 rpm or 12,000 rpm. The resultant nanoparticles were evaluated based on particle size, zeta potential, and entrapment efficacy. DMAB formulated NPs showed the lowest particle size (108 ± 2.1 nm) and highest zeta potential (-27.71 ± 0.6 mV) at 0.1 and 0.25% respectively, after centrifugation at 12,000 rpm. Results of the PVA based NP formulation showed the smallest particle size (92.4 ± 7.6 nm) and highest zeta potential (-11.14 ± 0.5 mV) at 0.25% and 1% w/v, respectively, after centrifugation at 12,000 rpm. Drug entrapment reached 77.3 ± 3.5% and 80.2 ± 1.2% efficiency with DMAB and PVA formulations, respectively. The results of our study indicate the use of DMAB for increased nanoparticle stability during formulation. Our study supports the effective utilization of PLGA based nanoparticle formulation for diclofenac.

Introduction

Over the past decade, there has been an increased interest in particle manipulation and nanosizing of selected drugs. In particular, polymeric nanoparticle formulation has gained an increasing amount of public attention in the fields of drug delivery and pharmaceutics. In recent years, the application of polymer based nanoparticles in drug formulation has garnered immense attention. Industry has focused, in large part, on the utilization of biodegradable polymer based nanoparticles as effective drug delivery agents because of their ability to prolong drug release, increase drug bioavailability, decrease drug degradation and reduce drug toxicity [1]. Research in nanoparticle drug formulations has focused heavily on the use of poly(lactic acid) (PLA), poly(D,L glycolide) (PLG), and poly(lactide-co-glycolide) (PLGA) (Fig. 3.1) based nanoparticles because of their tissue compatibility, low toxicity, and high rate of hydrolysis [2]

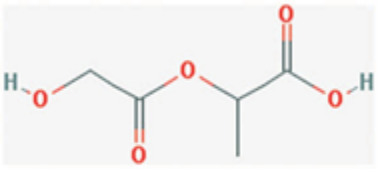

Figure 3.1 Chemical structure of poly (lactide-co-glycolide) (PLGA)

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed drugs in the world [3]. NSAIDs are pharmaceutical agents that exert analgesic and anti-inflammatory effects through the inhibition of the cyclooxygenase family of enzymes. Diclofenac is a NSAID that is commercially available in its sodium (Fig. 3.2) or potassium salt form [4].

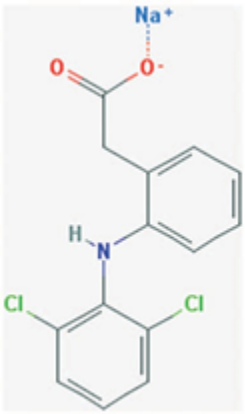


Figure 3.2 Chemical structure of diclofenac sodium

Like other NSAIDs, common side effects associated with the use of diclofenac include gastrointestinal lesion formation, and renal damage [4]. Interestingly, studies have shown a reduction in gastrointestinal and renal side effects associated with various drugs when encapsulated into polymer

based nanoparticles and administered orally [5-7]. These results demonstrate the effectiveness of nanoparticle formulation in reducing and/or eliminating potential adverse side effects associate with orally delivered toxic drugs.

Diclofenac nanoparticle reformulation has been used for ophthalmic and transdermal delivery with promising results [8-11]. The purpose of this study was to develop and characterize a new oral formulation of diclofenac using polymer based nanoparticles. Nanoparticles were synthesized using a solvent-evaporation technique and the effects of centrifugation speed and concentrations of two different stabilizers, poly (vinyl alcohol) (PVA) (Fig. 3.3) or didodecyldimethylammonium bromide (DMAB) (Fig. 3.4), was examined for effects on entrapment efficiency, particle size, and stability.

Figure 3.3 Chemical structure of poly vinyl alcohol (PVA

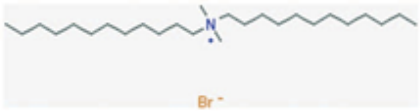


Figure 3.4 Chemical structure of didodecyldimethylammonium bromide (DMAB)

Materials and methods

2.1. Materials

PLGA (50:50 copolymer compositions; MW 30,000 Da), didodecyldimethylammonium bromide (DMAB), poly vinyl alcohol (MW 89,000 Da) and 15 mL Corning centrifuge tubes were purchased from Aldrich (St. Louis, MO, USA). Diclofenac was obtained from MP Biomedical (Solon, OH, USA). Ethyl acetate and HPLC grade water were purchased from Fischer Scientific Laboratory (Fair Lawn, NJ, USA). Phosphate buffer pH 7.0 was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). 0.2 micron syringe filters were obtained from Millipore Corporation (Carrigtwohill, Ireland).

2.2. Method of Nanoparticle Preparation

Nanoparticles were prepared by an emulsion – diffusion – evaporation technique [5] with slight modifications. Briefly, 45 mg of diclofenac and 50 mg of PLGA were placed in 3 mL ethyl acetate and stirred at 750 rpm for 30 minutes. Varying concentrations of PVA (0.1, 0.25, 0.5, or 1% w/v) or DMAB

(0.1, 0.25, 0.5, 0.75, or 1% w/v) stabilizers were placed within 6 mL of HPLC grade water heated to 140 °C and stirred at 750 rpm until fully dissolved. The organic phase was then added to aqueous phase in a drop wise manner under moderate stirring then sonicated for 5 minutes at 20 kHz using a sonic dismembrator (Fischer Scientific, Fair Lawn, NJ, USA). To facilitate diffusion, 25 mL of water was added to each emulsion under constant stirring at 750 rpm. Emulsions were stirred at 750 rpm for 4 hours to insure complete organic phase evaporation. After which, each emulsion was centrifuge (8,800 rpm or 12,000 rpm) and supernatant was collected.

2.3. Particle Size and Zeta Potential

Particle size was measured by dynamic light scattering using a Nicomp particle sizer (Particle Sizing Systems, Port Richy, FL, USA). Zeta potential was estimated on the basis of electrophoretic mobility under an electrical field. All measurements were performed in triplicates.

2.4. Entrapment Efficiency

To measure the amount of diclofenac nanoparticle entrapment, the amount of diclofenac present within solutions following end stage centrifugation was calculated. Diclofenac stock solution dissolved in methanol (200 mg/mL) was used to construct a standard calibration curve (10,000 – 2,000,000 ng/mL). Pure methanol was used as a blank experiment before UV measurement, after which total NP drug content was calculated using the standard curve after control for blank NPs. Quantification was performed by UV-spectrophotometry (Eppendorf Biophotometer, Hauppauge, NY, USA) with absorbance set at 280 nm. Entrapment efficiency was calculated using the following equation: Entrapment Efficiency = (Amount of diclofenac entrapped within nanoparticles/Total amount of diclofenac used for synthesis) X 100

2.5. Effects of Centrifugation Speed and Stabilizer Concentration on Nanoparticle Properties

NPs were formulated with five different concentrations of DMAB (0.1, 0.25, 0.5, 0.75, or 1 % w/v) and four different concentrations of PVA (0.1, 0.25, 0.5, or 1% w/v). Effect of stabilizer concentrations and two centrifugation rates (8,800 or 12,000 rpm) on zeta potential, particle size, and entrapment efficiency was evaluated.

2.6. Nanoparticle Morphology Characterization

Shape and surface morphology of NPs were examined with a transmission electron microscope (TEM) (Tecnai Philips Transmission Electron Microscope; FEI, Hillsboro, Oregon, USA). NP solutions were vortex mixed and 2 µL of suspension was placed on a 100 mesh copper grid covered with Formvar film (Electron Microscopy Sciences, Hatfield, Pennsylvania). Samples were kept under ventilation for 2 hours to allow for complete drying, than examined by TEM at 80 kV.

2.7. In Vitro Drug Release Study

In vitro release of diclofenac sodium was carried out as previously described with slight modification [12,13]. Briefly, 2 mL of solution containing diclofenac formulated nanoparticles were placed into 15 mL centrifuge tubes containing 8 mL phosphate buffer. Suspensions were then placed on an electronic shaker set at 100 rpm. At various time points, 2 mL of release medium was removed and replaced with the same volume of fresh medium. Isolated samples were centrifuged at 4,400 rpm for 5 minutes and filtered through a 0.2 micron syringe filter. Analysis was carried out using a UV spectrophotometer set at 280 nm with empty nanoparticle solutions used as control.

2.8. Data treatment

Data is represented as mean ± standard deviation (SD). The unpaired Student's *t*-test was used to analyze cumulative release data for identical stabilizer concentrations.

Results

3.1. Synthesis and assembly of diclofenac loaded PLGA based nanoparticles

The synthesis of PLGA based nanoparticles was achieved through an emulsion – diffusion – evaporation technique. A solution of diclofenac and PLGA dissolved in ethyl acetate was added to an aqueous solution containing stabilizer in a drop wise manner, followed by sonication and moderate stirring for 4 hours to ensure complete organic phase evaporation. The synthesis of PLGA polymer based nanoparticles using ethyl acetate as the primary solvent has been reported before [5,14,15]. In the present study, PLGA NPs containing diclofenac were prepared using DMAB and PVA as stabilizers. To determine optimal nanoparticle production, varying levels of DMAB and PVA stabilizer concentration along with varying centrifugation speeds were evaluated in the determination of peak nanoparticle synthesis (Table 3.1). Aqueous to organic phase ratios of 1:1 were found to elicit particle aggregation during formulation process (data not shown). As a result, a direct 1:2 ratio of organic to aqueous phase solution was used for nanoparticle synthesis.

Table 3.1

Method of Nanoparticle Preparation

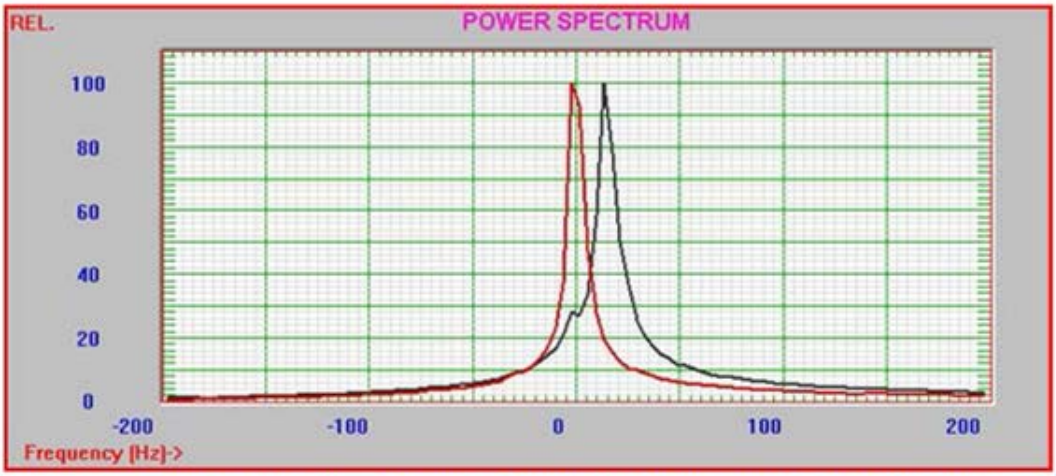
	Ingredients	Amount
Organic Phase	PLGA	50 mg
	Ethyl Acetate	3 mL
	Diclofenac	45 mg
Aqueous Phase	DMAB	Variable[1]
	PVA	Variable[2]
	HPLC grade H2O	6 mL
Emulsifier	Sonic Dismembrator	5 minutes (25 kHz)

[1]DMAB concentrations varied 0.1, 0.25, 0.5, 0.75, and 1% w/v with respect to solvent

[2]PVA concentrations varied 0.1, 0.25, 0.5, and 1% w/v with respect to solvent

3.2. Influence of centrifugation and DMAB stabilizer on nanoparticle size and stability

Particle size and zeta potential measurements were conducted using a NICOMP Zeta Sizer System with DMAB formulated polymer NPs (Fig. 3.5).



Measurements revealed low particle size and increased zeta potential stability with low stabilizer concentrations. Zeta potential reached peak measurements at 0.1 and 0.25% DMAB concentration. A maximum zeta potential was reached at -27.7 ± 0.6 mV using 0.25% DMAB formulation (Table 3.2). Particle size was lowest using 0.1% DMAB concentrations and highest at 0.5 and 0.75% DMAB concentrations (Table 3.2). Interestingly, centrifugation speed was found to positively affect stability and particle size. As centrifugation speed was increased from 8,800 rpm to 12,000 rpm, there was a further increase in zeta potential and decrease in particle size when compared to lower centrifugation

speed. Stability and particle size still followed the same trends as seen in lower centrifugation speeds in relation to stabilizer concentration with the exception of 0.25 and 0.5%, which showed an increase in zeta potential and reduction in particle size (Table 3.2).

Table 3.2
Effect of DMAB stabilizer and centrifugation speed on mean particle size and zeta potential of nanoparticles

Centrifugation Speed (rpm)	Concentration (% w/v)	Zeta Potential* (mV)	Particle Size* (nm)
8,800	0.1	-21.2 ± 1.5	132.0 ± 3.6
	0.25	-11.8 ± 0.9	214.0 ± 1.5
	0.5	-7.4 ± 0.4	216.0 ± 3.4
	0.75	-12.7 ± 0.9	182.6 ± 6.8
	1	Particle Aggregation	Particle Aggregation
12,000	0.1	-21.6 ± 0.6	108.0 ± 2.1
	0.25	-27.7 ± 0.6	168.0 ± 2.2
	0.5	-21.3 ± 0.9	158.6 ± 4.8
	0.75	-13.6 ± 2.1	183.9 ± 4.9
	1	Particle Aggregation	Particle Aggregation

All values reported as mean ± SD (n = 3)
*Average triplicate measurement

3.3. Influence of centrifugation and PVA stabilizer on nanoparticle size and stability

Measurements of NP formulated nanoparticles using PVA stabilizer revealed lower stability and lower particle size parameters in comparison to DMAB formulations (Table 3.3). At 8,800 rpm centrifugation speed, particle size and zeta potential showed inverse trends in relations to stabilizer concentration. As stabilizer was increased zeta potential decreased, conversely particle size increased with increasing stabilizer concentrations. Higher centrifugation speeds maintained similar patterns with the exception of 0.25 and 1% PVA concentrations (Table 3.3). Formulations at 0.25% showed a slight reduction in particle size, reaching its lowest diameter at 92.4 ± 7.6 nm. Also, 1% stabilizer formulations showed a higher degree of stability with increasing zeta potential, reaching a peak zeta potential of -11.1 ± 0.5 mV (Table 3.3).

Table 3.3

Effect of PVA stabilizer and centrifugation speed on mean particle size and zeta potential of nanoparticles

Centrifugation Speed (rpm)	Concentration (% w/v)	Zeta Potential (mV)	Particle Size (nm)
8,800	0.1	-6.7 ± 2.8	103.0 ± 10.6
	0.25	-5.6 ± 2.7	114.9 ± 12.7
	0.5	-4.3 ± 1.1	119.2 ± 11.6
	1	-4.2 ± 0.9	129.4 ± 2.4
12,000	0.1	-7.4 ± 0.9	94.1 ± 12.6
	0.25	-7.0 ± 2.1	92.4 ± 7.6
	0.5	-4.9 ± 2.2	113.5 ± 22.9
	1	-11.1 ± 0.5	120.5 ± 6.4

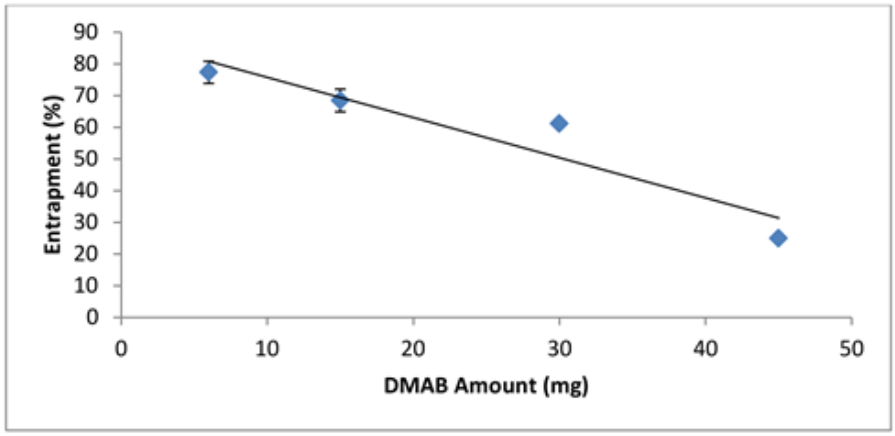
All values reported as mean ± SD (n = 3)

*Average triplicate measurement

3.4. Effects of stabilizer concentrations on diclofenac entrapment

Amount of drug entrapment was determined by UV-spectroscopy in varying stabilizer concentrations. DMAB formulated NPs reached peak entrapment at low w/v concentration. Entrapment levels with DMAB reached as high as 77.3 ± 3.5% and were seen at 0.1% w/v DMAB concentrations. When the concentration of DMAB increased, a linear reduction in overall drug entrapment and entrapment amounts was seen (Fig. 3.6A) (Table 3.4).

A.



B.

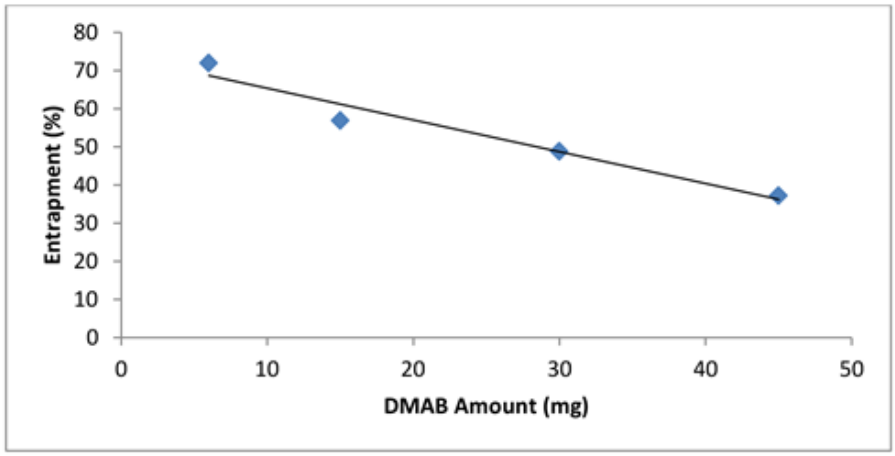


Figure 3.6 Entrapment effects of varying DMAB stabilizer concentrations. Entrapment efficiency after 8,800 rpm centrifugation of diclofenac loaded NPs (A) and entrapment efficiency after 12,000 rpm centrifugation of diclofenac loaded NPs (B).

Conversely, as centrifugation speed was increased, slightly lower levels of drug entrapment were obtained for each formulation. Linear regression in overall drug entrapment percentages were still

maintained (Fig. 3.6B) (Table 3.4).

Table 3.4

Entrapment efficiency of diclofenac loaded NPs using DMAB stabilizers at varying concentration

Centrifugation Speed (rpm)	Concentration (% w/v)	Amt. Entrapped* (mg)	EE* (%)
8,800	0.1	34.8 ± 1.7	77.3 ± 3.5
	0.25	30.8 ± 1.6	68.4 ± 3.6
	0.5	27.5 ± 0.2	61.1 ± 0.1
	1	11.2 ± 0.1	24.9 ± 0.1
12,000	0.1	32.4 ± 0.2	71.9 ± 0.4
	0.25	25.6 ± 0.2	56.8 ± 0.4
	0.5	21.9 ± 0.3	48.8 ± 0.1
	1	16.7 ± 0.1	37.2 ± 0.2

All values reported as mean ± SD (n = 3). EE – Entrapment efficiency. Amount entrapped per 45 mg diclofenac. *Average triplicate measurement

Measurements of drug entrapment utilizing PVA stabilizers showed similar findings to DMAB formulations. Drug entrapment levels reached 73.6 ± 0.9% and 75.2 ± 1.7% entrapment for PVA formulations at 0.25 and 0.5% w/v (Table 3.5). When centrifugation speed was increased, drug entrapment of diclofenac reached 80.2 ± 1.2% entrapment at a lower 0.1% PVA formulation (Table 3.5). Increases in centrifugation speed increased drug entrapment at 0.1%, 0.25% and 1% PVA concentrations. Drug entrapment efficiency reduced from 75.2 ± 1.7% to 28.6 ± 1.9% in 0.5% PVA formulations when speed in centrifugation was increased (Table 3.5).

Table 3.5

Entrapment efficiency of diclofenac loaded NPs using PVA stabilizers at varying concentration

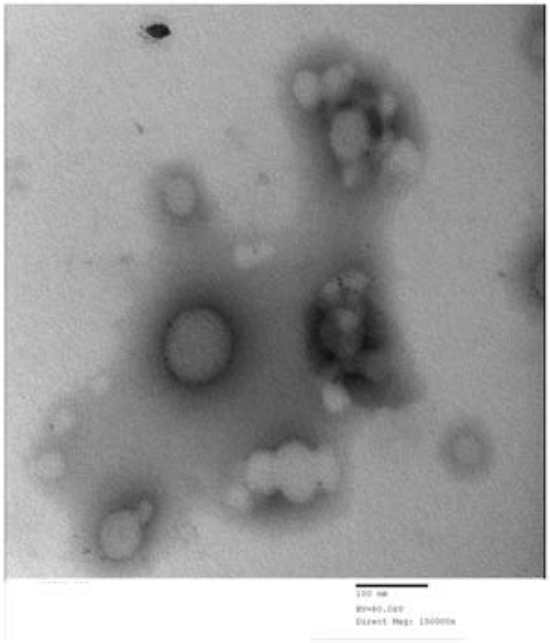
Centrifugation Speed (rpm)	Concentration (% w/v)	Amt. Entrapped* (mg)	EE* (%)
8800	0.1	31.7 ± 0.4	70.3 ± 1.1
	0.25	33.1 ± 0.4	73.6 ± 0.9
	0.5	33.9 ± 0.9	75.2 ± 1.7
	1	29.9 ± 0.6	66.4 ± 1.2
12000	0.1	36.1 ± 0.5	80.2 ± 1.2
	0.25	34.7 ± 0.2	77.1 ± 0.6
	0.5	14.5 ± 0.9	28.6 ± 1.9
	1	31.4 ± 0.4	69.8 ± 0.2

All values reported as mean ± SD (n = 3). EE – Entrapment efficiency. Amount entrapped per 45 mg diclofenac. *Average triplicate measurement

3.5. Nanoparticle Shape and Surface Morphology

Morphology studies were carried out using 0.25% DMAB and 1% PVA concentrations. These stabilizer concentrations were chosen based on zeta potential and nanoparticle stability characteristics. The TEM images of blank and diclofenac loaded DMAB (Fig. 3.7A and 3.7B, respectively) and PVA (Fig. 3.8A and 3.8B, respectively) formulated NPs support the particle size data obtained by our characterization studies performed with the zetasizer. DMAB formulated NPs have a distinct, spherical shape composed of a dense core with diclofenac loaded NPs showing a slightly increased size diameter due to drug incorporation (Fig. 3.7B). Drug incorporation did not affect overall particle shape. Morphology of PVA formulated NPs show a high degree of shape variation and aggregation in both blank NPs (Fig. 3.8A) and diclofenac loaded NPs (Fig. 3.8B).

A.



B.

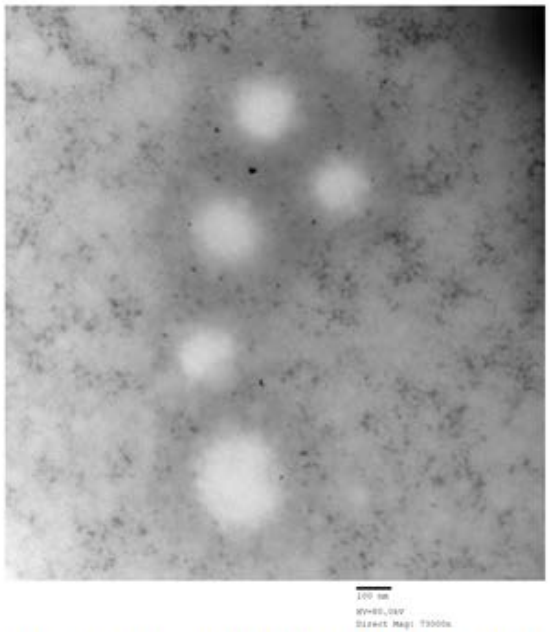
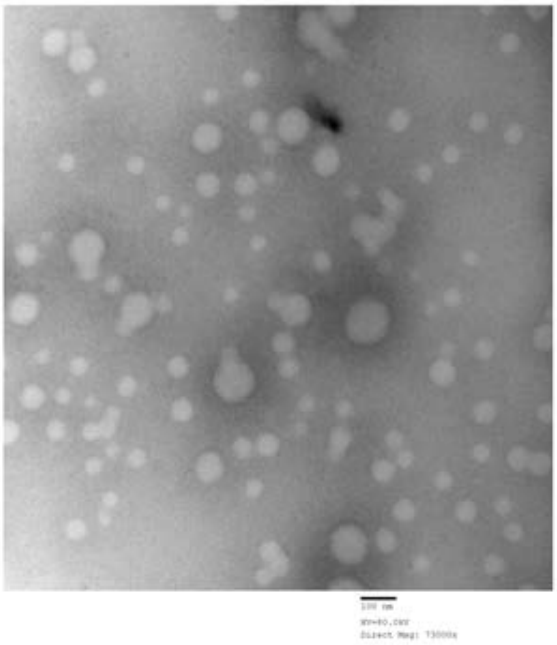


Figure 3.7 Morphological analysis of 0.25% DMAB formulated NPs. Transmission electron microscopy image of empty NPs formulated with 0.25% DMAB stabilizer (A) and transmission electron microscopy image of diclofenac loaded NPs formulated with 0.25% DMAB stabilizer (B).

A.



B.

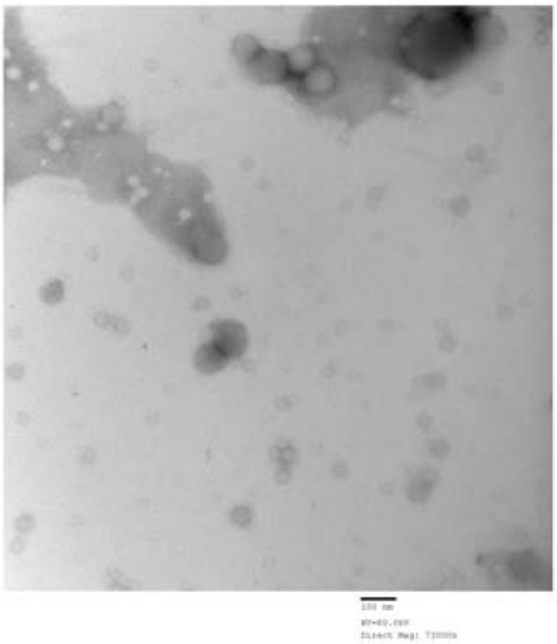


Figure 3.8 Morphological analysis of 1% PVA formulated NPs. Transmission electron microscopy image of empty NPs formulated with 1% PVA stabilizer (A) and transmission electron microscopy image of diclofenac loaded NPs formulated with 1% PVA stabilizer (B).

3.6. Stabilizer Influence on In Vitro Diclofenac Release

In vitro release studies were performed on two different stabilizer concentrations for both DMAB and PVA formulated NPs. Stabilizer concentrations of 0.1% and 0.25% centrifuged at 12,000 rpm were chosen based on their efficient level of drug entrapment and best fit mean representation of particle

stability of each stabilizer group. The *in vitro* release of both DMAB and PVA formulated diclofenac loaded NPs are given in Figs. 3.9 and 3.10.

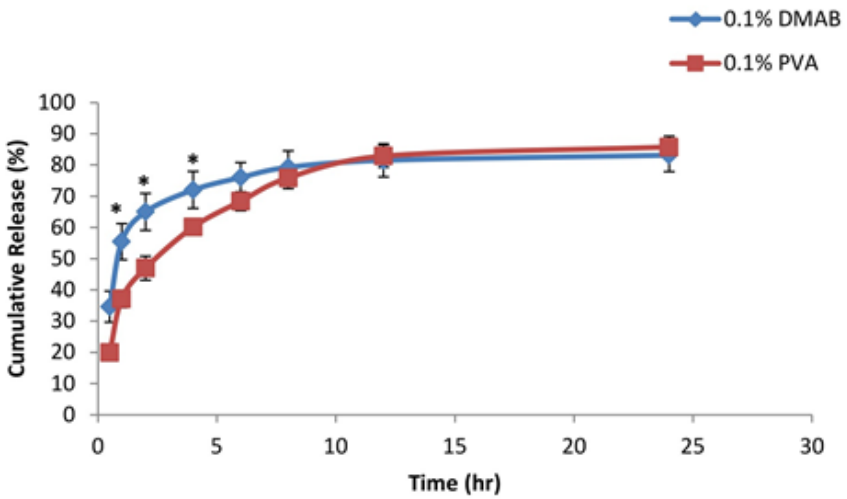


Figure 3.9 *In vitro* drug release study with 0.1% stabilizer concentrations. *In vitro* release profile of diclofenac sodium in phosphate buffer of pH 7 from 0.1% PVA formulated NPs and 0.1% DMAB formulated NPs (mean ± SD, n = 3, p 0.05)

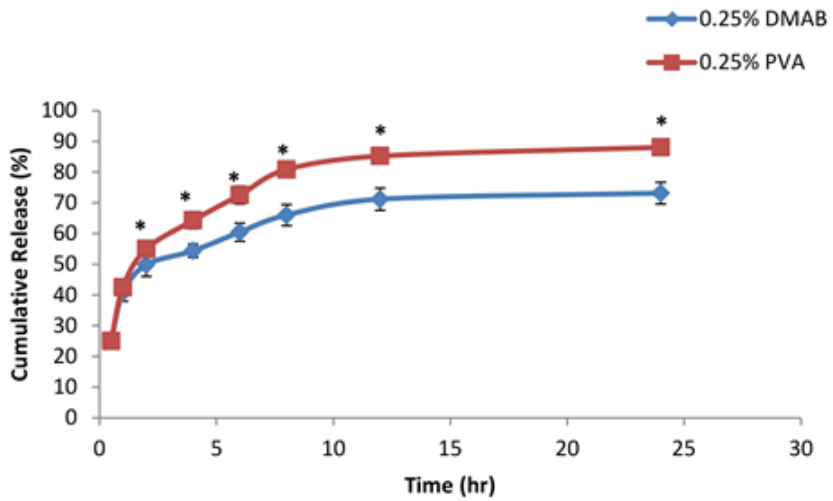


Figure 3.10 *In vitro* drug release study with 0.25% stabilizer concentrations. *In vitro* release profile of diclofenac sodium in phosphate buffer of pH 7 from 0.25% PVA formulated NPs and 0.25% DMAB formulated NPs (mean ± SD, n = 3, p 0.05)

The statistical comparison of the percentage drug release values obtained with the different nanoparticle stabilizer compositions at specific sampling times revealed significant difference ($P < 0.05$) in both stabilizer concentration groups. DMAB formulations at 0.1% showed an initial significant increase in drug release in comparisons to 0.1% PVA formulations during the initial 4 hr time frame (Fig. 3.9) ($P < 0.05$). After 24 hrs, total drug release was similar with a cumulative release of over 80% achieved for both groups (Fig. 3.9). The drug release of NPs formulated with 0.25% PVA showed a similar pattern of initial release of diclofenac in comparison to DMAB formulation (Fig.3.10). Both formulations experienced greater than 40% release during the first hour of the study. However, after the first initial

hour, cumulative release began to increase significantly in PVA formulated groups at each successive time point ($P < 0.05$). PVA formulations reached an average cumulative release percentage of 88%, while DMAB formulation reached an average cumulative release of 73% (Fig. 3.10).

Discussion

The adverse cardiovascular, gastrointestinal, and renal side effect caused by NSAID consumption has restricted the clinical use of these important drugs. The main objective of our research was to reformulate and develop a new nanoparticle formulation for diclofenac sodium that could replace traditional capsule and tablet oral administration and minimize or delay the onset of adverse side effects commonly associated with NSAIDs. Nanoparticles have been used for the development of a variety of different drug delivery systems [1,14,16-20]. Recent studies have shown the use of polymer based nanoparticles in the reformulation of diclofenac for ophthalmic and colonic us with promising results [13,21-23]. Topically formulated diclofenac delivery systems have also been used with success for the treatment of a variety of inflammatory skin diseases [24-26]. Diclofenac delivery utilizing small lipid nanoparticles showed promising results in the realm of drug reformulation and enhanced drug delivery systems [27,28]. Interestingly, the use of microspheres in reformulation has demonstrated enhanced drug entrapment and drug release of diclofenac [29]. One study showed that the use of Eudragit and alginate polymer systems improved drug release profiles and enhanced the physical properties of tablet compaction [30], while other studies have demonstrated a high degree of stability and morphology in microsphere development with the use of PVA [31,32]. However, to date, reformulation characteristics of diclofenac nanoparticles for oral delivery has yet to be extensively examined.

In this study, diclofenac loaded PLGA NPs were formulated following an emulsion – diffusion – evaporation technique using DMAB or PVA as stabilizers. Stabilizers function as emulsifying agents that can offset the surface tension between organic and aqueous phases, thereby increasing drug solubility and nanoparticle encapsulation. Because of this understanding, a variation in the level of stabilizer used can equate to variations in nanoparticle characteristics during the formulation process [5,33-36]. In our study, we formulated drug loaded NPs at varying levels of PVA and DMAB stabilizer concentrations to elucidate the most efficient formulation characteristics for maximum drug encapsulation, stability and size.

A study conducted by Cetin *et al.* [12] demonstrated low levels of diclofenac NP stability and entrapment efficiency when using a Eudragit®L100 and Eudragit®L100 PLGA based nanoparticle formulation with PVA as stabilizer. Consequently, they also showed that variations in polymer concentrations did not effectively alter NP characteristics to a measurable degree. Based on these

findings diclofenac formulated NPs appeared to offer complications in achieving premium NP characteristics during formulation [12]. In our study, drug loaded NPs were prepared at varied DMAB (0.1, 0.25, 0.5, 0.75, or 1% w/v) and PVA (0.1, 0.25, 0.5, or 1% w/v) concentrations. Nanoparticle size was at its largest when DMAB concentrations were between 0.25, 0.5, and 0.75% w/v. Consequently, zeta potential and stability of NPs were highest when DMAB concentrations were lower (Table 2). Surprisingly, our study demonstrated diclofenac loaded NP particle sizes of 108 and 92.4 nm with DMAB and PVA, respectively. Zeta potential stability measurements reached as high as -27.7 ± 0.6 mV in formulations using DMAB, and were substantially lower in formulation utilizing PVA stabilizers (Tables 3.2 and 3.3). These results are further supported by previously published findings in which a Eudragit®RS100 based formulation of diclofenac was used for nanoparticle characterization. It was found that variations in Eudragit concentrations effectively altered drug entrapment and particle diameter characteristics for diclofenac loaded NPs. Alterations in diclofenac to Eudragit concentrations resulted in variable measurements in particle diameter, ranging in size from 103 ± 6 to 170 ± 36 nm, which are consistent with the size variations of 92.4 ± 7.6 to 216 ± 3.4 nm found within our study [37]. Our morphological analysis showed distinct, well defined diclofenac loaded NPs when formulated with 0.25% DMAB stabilizer (Fig. 3.7B). The visualization of 1% PVA formulations showed distinct NP aggregation (Fig. 3.8A and 3.8B). These findings are consistent with particle properties of low zeta potential noticed during our characterization studies performed with the zetasizer. A more pronounced zeta potential value has a tendency to stabilize and prevent particle aggregation [38]. It is known that particles with a larger charge experience a much higher degree of repulsion from other like charged particles [38]. The high degree of particle aggregation of 1% PVA formulations are indicative of poor stability and reduced zeta potential [38,39], which is in line with our initial findings. TEM scaling measured particle sizes within the range reported by zetasizer analysis for both formulations (Table 3.2 and 3.3). These findings suggest the use of specific DMAB concentrations in effectively formulating stable PLGA based diclofenac loaded NPs.

Entrapment efficiency is a crucial step in the characterization of an effectively formulated drug encapsulated nanoparticle. In our study, the result of drug encapsulation efficiencies with differing stabilizer concentrations and centrifugation speeds is shown in Tables 3.4 and 3.5. Our study showed high degrees of drug encapsulation for both DMAB and PVA formulations. In DMAB formulations, drug encapsulation followed a linear decline in the amount of drug entrapped in relation to the amount (Figs. 3.6A and 3.6B) or concentration (Table 3.4) of stabilizer used. The highest level of entrapment reached was 77.3 ± 3.5% and was seen with DMAB concentrations of 0.1% w/v. It is important to note that stabilizing agents are important factors in determining the entrapment efficiency of lipophilic drugs.

Stabilizers function by forming molecular micelles through interactions between hydrophobic portions of the stabilizers with the hydrophobic core of the NP. In other studies, it was shown that as concentrations of DMAB increases, entrapment of lipophilic drug increases in response [5]. Our findings have demonstrated the opposite in regards to entrapment, suggesting that the high polarizability of diclofenac effectively works against the micelle formation properties of DMAB resulting in a reduction in drug entrapment as DMAB concentrations increase.

Measurements of entrapment efficiency in formulations utilizing PVA as stabilizer showed similar results to those obtained with DMAB. An entrapment efficiency of 80.2 ± 1.2% was seen at PVA concentrations of 0.1% following centrifugation at 12,000 rpm (Table 3.5). Interestingly, while entrapment efficiency remained high, zeta potential measurements remained close to zero, indicating low levels of stability (Table 3.3). Two possible explanations of our findings exist. One possibility is the presence of residual PVA. The presence of residual PVA on the nanoparticle surface has been found to mask charged groups existing on the surface of PVA formulated nanoparticle [40]. Thus, residual PVA may effectively create a shield between the nanoparticle and its surrounding medium, resulting in lower zeta potential measurements that still maintain higher levels of entrapment [40,41]. A second possibility is the correlation between zeta potential and nanoparticle stability. Zeta potential measurements closer to zero represents a high degree of non-stability with a weak surface charge surrounding the NP. It is highly possible that NPs degrade and break during the centrifugation process, in turn causing entrapped drug to leak from the NP into the medium. The leakage of free drug into the medium could result in higher levels of spectrophotometric drug detection during entrapment studies.

Results of our *in vitro* study showed an increased initial diclofenac burst release for NPs formulated with 0.1% DMAB when compared to 0.1% PVA (Fig.3. 9). Inverse results were seen with stabilizer concentrations at 0.25%. Formulations with PVA at 0.25% concentration demonstrated a marked increase in drug release following one hour of agitation when compared to 0.25% DMAB formulation. Drug release from nanoparticles can occur through several means such as desorption of drug close to the surface of the nanoparticle, diffusion through the polymer matrix, or matrix erosion [2]. The fast release of diclofenac in 0.1% DMAB concentrations may be due to diclofenac polarity and increased levels of diclofenac absorbed closer to the surface of our DMAB nanoparticles [42]. The stunted release noticed in 0.25% DMAB formulation could be attributed to increased electrostatic adhesion of the drug molecules to the polymeric matrix. It has been shown that particles with larger zeta potential demonstrate higher adhesion of drug molecules to the polymeric matrix as a result of electrostatic adhesion [2]. It is possible that adhesion may be taking place within these particles that may

reduce diffusion of diclofenac within the PLGA nanoparticle core after exposure to dissolution medium [42,43].

The purpose of our study was to elucidate a novel formulation for diclofenac sodium using polymer based nanoparticles. Our results are based on NP formulations using two different stabilizers at varying concentrations at two distinct centrifugation speeds. As such, our results are limited to NP characteristics utilizing PVA and DMAB stabilizers. It is entirely possible that the use of other stabilizing agents could result in alterations of NP characteristics above what has been seen in our study.

Solvents play a critical role in the determination of NP characteristics as well. In our study, we utilized ethyl acetate as our primary organic solvent for NP preparation. Our choice of solvent was based on evidence seen in previous publications which utilized ethyl acetate in conjunction with other solvents on the determination of NP characteristics. Ethyl acetate was shown to be most effective at creating stable NPs in conjunction with the use of PLGA and DMAB as stabilizer [5,44]. The use of differing solvents would alter pH characteristic of formulation medium. Since our focus was on the salt form of diclofenac it is possible that alteration in organic solvents could alter ionization and solubility of diclofenac sodium, leading to differences in particle size, stability and entrapment.

Conclusions

In summary, our findings revealed the fact that diclofenac loaded PLGA NPs could be prepared utilizing low concentrations of PVA and DMAB stabilizers. Formulation was achieved through a very basic and simple evaporation – diffusion technique utilizing ethyl acetate as organic solvent. In comparisons to previous reports, the NPs of diclofenac developed in this study provided adequate diclofenac entrapment levels and showed superior levels of stability with a marked reduction in overall particle size. Diclofenac loaded PLGA NPs could be used as an alternative to existing oral delivery methods and aid in offsetting deleterious side effects common to NSAID use.

Acknowledgments

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Figure Legends

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CHAPTER 4

Effect of Formulation Variables on Preparation of Celecoxib Loaded Polylactide-Co-Glycolide Nanoparticles

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Abstract

Polymer based nanoparticle formulations have been shown to increase drug bioavailability and/or reduce drug adverse effects. Nonsteroidal anti-inflammatory drugs (e.g. celecoxib) reduce prostagladin synthesis and cause side effects such as gastrointestinal ulcerations and renal complications. The aim of this study was to formulate celecoxib entrapped poly lactide-co-glycolide based nanoparticles through a solvent evaporation process using didodecyldimethylammonium bromide or poly vinyl alcohol as stabilizer. Nanoparticles were characterized for zeta potential, particle size, entrapment efficiency, and morphology. Effects of stabilizer concentration (0.1, 0.25, 0.5, and 1 % w/v), drug amount (5, 10, 15, and 20 mg), and emulsifier (lecithin) on nanoparticle characterization were examined for formula optimization. The use of 0.1%, 0.25% and 0.5% w/v didodecyldimethylammonium bromide resulted in more than a 5-fold increase in zeta potential and more than a 2 fold increase in entrapment efficiency with a reduction in particle size over 35%, when compared to stabilizer free formulation. Nanoparticle formulations were also highly influenced by emulsifier and drug amount. Using 0.25% w/v didodecyldimethylammonium bromide NP formulations, peak zeta potential was achieved using 15 mg celecoxib with emulsifier (17.15 ± 0.36 mV) and 20 mg celecoxib without emulsifier (25.00 ± 0.18 mV). Peak NP size reduction and entrapment efficiency was achieved using 5 mg celecoxib formulations with (70.87 ± 1.24 nm and 95.55 ± 0.66%, respectively) and without (92.97 ± 0.51 nm and 95.93 ± 0.27%, respectively) emulsifier. In conclusion, formulations using 5 mg celecoxib with 0.25% w/v didodecyldimethylammonium bromide concentrations produced nanoparticles exhibiting enhanced size reduction and entrapment efficiency. Furthermore, emulsifier free formulations demonstrated improved zeta potential when compared to formulations containing emulsifier ($p < 0.01$). Therefore, our results suggest the use of emulsifier free, 5 mg celecoxib drug formulations containing 0.25% w/v didodecyldimethylammonium bromide for production of polymeric NPs that demonstrate enhanced zeta potential, small particle size, and high entrapment efficiency.

Keywords: NSAIDs; Celecoxib; Formulation; PLGA; Nanoparticles; DMAB; PVA

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are well established for the treatment of pain and inflammation. They function by acting on the cyclo-oxygenase (COX) family of enzymes and inhibiting the conversion of arachidonic acid to prostaglandins and thromboxanes [1-4]. The COX enzyme exists as at least two different isozymes, COX-1 and COX-2. The COX-1 enzyme is constitutively expressed in most tissue and functions to regulate hemodynamics and maintain gut integrity. COX-2 is an inducible enzyme found primarily at sites of inflammation that mediates fever and pain [5-7]. COX-2 has been found to be constitutively expressed in certain tissue such as the kidneys, the reproductive tract, and gastric mucosa [2,8-11]. Traditionally, NSAIDs function by inhibiting both COX-1 and COX-2 isozymes and provide analgesic and anti-inflammatory benefits. These benefits are thought to arise primarily from the inhibition of COX-2, while the adverse effects (e.g. ulceration) were thought to occur from over inhibition of COX-1 [12-14]. As a result, COX-2-selective inhibitors (COXIBs) were developed to provide analgesic and anti-inflammatory benefits, while minimizing the gastrointestinal adverse side effects associated with traditional NSAID use [1,12].

Celecoxib (CEL) is a COXIB used in the treatment of pain and inflammation [13-15]. Evidence suggests that CEL use effectively reduces clinical gastrointestinal events in comparison to other NSAIDs, making it one of the most commonly prescribed COX-2 specific inhibitors [16-18]. Despite the general safety of CEL in regards to gastrointestinal tolerability, its use has been associated with the development of several adverse side effects including cardiovascular events, and renal toxicity [18,19]. Many CEL delivery systems designed to help reduce and alleviate the formation of CEL associated side effects have been developed [20-23]. Studies utilizing nanoparticle (NP) formulations have shown promising results in overcoming high dose oral administration of CEL [22,24-26]. One study showed enhanced drug retention at the site of action following intra-articular injection of small lipid nanoparticle formulated CEL in the treatment of joint pain [27]. Another study showed enhanced anti-inflammatory effects of

CEL utilizing NP formulated transdermal drug delivery [28]. A third study showed enhanced inhibition of tumor growth with a reduction in side effects using hydroxyapatite-chitosan nanocomposited CEL in the treatment of colon cancer [29].

Polymer based NPs are commonly used to improve drug bioavailability and/or reduce drug associated side effects [30]. Poly lactide-co-glycolide (PLGA) is a polymer that has been commercialized for a variety of drug delivery systems and is frequently used in the design of biocompatible NPs [31]. PLGA is approved by the Food and Drug Administration as a biodegradable polymer that degrades to the nontoxic tricarboxylic acid cycle intermediates, lactic acid and glycolic acid [31-33]. Use of PLGA based NPs for enhanced delivery of CEL has been met with a variety of different results. [20,21]. However, known NP stabilizers such as didodecyldimethylammonium bromide (DMAB) and poly vinyl alcohol (PVA) have yet to be used in the development of CEL loaded PLGA-NPs.

Previous studies have shown effective use of DMAB and PVA for formulation of small, highly entrapped NPs [34,35]. The aim of this study was to characterize and optimize CEL loaded PLGA-NPs by examining the influence of varying DMAB and PVA concentrations on NP characterstics. The effect of drug amount and emulsifier (lecithin) on zeta potential, particle size, entrapment efficiency, and morphology was also examined.

Materials and methods

1.1. Materials

DMAB, PVA (MW 89,000 – 98,000 Da, 99.9+% hydrolyzed), PLGA (50:50 copolymer compositions; MW 30,000 – 60,000 Da), and lecithin (99% phosphatidylcholine) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CEL base powder was obtained from Biovision Incorporated (Milpitus, CA,

USA). Acetone, ethyl acetate, and *high-performance liquid chromatography (HPLC)*-grade water were purchased from Fischer Scientific Laboratory (Fair Lawn, NJ, USA).

1.2. Preparation of CEL loaded PLGA-NPs

Formulation of NPs was carried out using a previously described solvent evaporation technique [34,36]. CEL-loaded NPs were formulated by dissolving 20 mg of CEL and 50 mg PLGA into 3 mL of ethyl acetate. The solution was stirred for 30 minutes at 750 rpm. Afterwards, 30 mg of lecithin was added to the organic solution followed by addition of 500 µL acetone as co-solvent. A varying range of DMAB or PVA concentrations (0.1%, 0.25%, 0.5%, and 1% w/v) was dissolved in 6 mL of *HPLC* grade water. Organic phase was then added to aqueous phase in a drop wise manner under moderate stirring followed by sonication for 5 minutes at 20 KHz. After sonication, solutions were stirred at 750 rpm for 1 hour to evaporate organic phase. Emulsions were then centrifuged at 12,000 rpm followed by separation of supernatant from precipitants. Additional NP formulations for optimization studies were carried out with 0.25% w/v DMAB concentration. Using the previously described process, emulsifier free CEL loaded PLGA-NPs were formulated with the exclusion of lecithin, while NP preparation looking at effects of drug amount was carried out using various amounts of CEL (5, 10, 15, and 20 mg).

1.3. Particle size and zeta potential

Intensity weighted mean particle size (diameter) was measured in triplicate by dynamic light scattering using a NICOMP particle sizer (Particle Sizing Systems, Port Richy, FL, USA). Zeta potential was estimated on the basis of electrophoretic mobility under an electrical field.

1.4. Drug entrapment

To measure drug entrapment efficiency 100 µL NP solution was added to 300 µL acetonitrile and vortex mixed for 30 seconds. Afterwhich, 100 µL of drug loaded NP solution was analyzied under *ultraviolet–visible spectroscopy* (Eppendorf Biophotometer, Hauppauge, NY, USA) at 260 nm using empty NPs solutions as blank. A standard calibration curve (50,000 – 2,000,000 ng/mL) was constructed using

titrated dilutions of CEL stock solution dissolved in acetonitrile. Drug entrapment efficiency (EE) was calculated using the following equation:

Entrapment efficiency = (Amount of CEL entrapped within nanoparticles/Total amount of CEL used for formulation) X 100

1.5. Morphology

Transmission electron microscopy (TEM) (Tecnai Philips Transmission Electron Microscope; FEI, Hillsboro, Oregon, USA) was used for evaluation of CEL loaded PLGA-NP shape and surface morphology. NP emulsions were vortex mixed and 2 µL of suspension was placed on a 200 mesh copper grid covered with Formvar film (Electron Microscopy Sciences, Hatfield, Pennsylvania). Samples were air dried for 1 hour then examined at 80 kV.

1.6. Stability of CEL loaded PLGA-NPs

CEL loaded PLGA-NP emulsions (5 mL) formulated at various drug amounts (5, 10, 15, and 20 mg) with or without emulsifier (0.25% w/v DMAB) were stored at 4 °C for a period of 16 weeks. After 16 weeks samples were removed from storage and analyzed for particle size, zeta potential, and drug entrapment efficiency. Particle characteristics were evaluated as previously described.

1.7. Data analysis

All experiments were performed in triplicate. NP characteristic data is represented as mean ± standard deviation (SD). A Student's t-test was used for comparison of two groups.

Results and discussion

1.8. Effect of stabilizer concentration on NP characteristics

CEL encapsulated PLGA-NPs were developed using lecithin as an emulsifier with DMAB or PVA (Table 4.1). The use of DMAB or PVA resulted in formation of CEL loaded PLGA-NPs with surface characteristics that displayed positive and negative charges, respectively. Because of the cationic

properties of DMAB [37-39], NPs formulated with inclusion of DMAB showed highly positive surface charges (Fig. 4.1A).

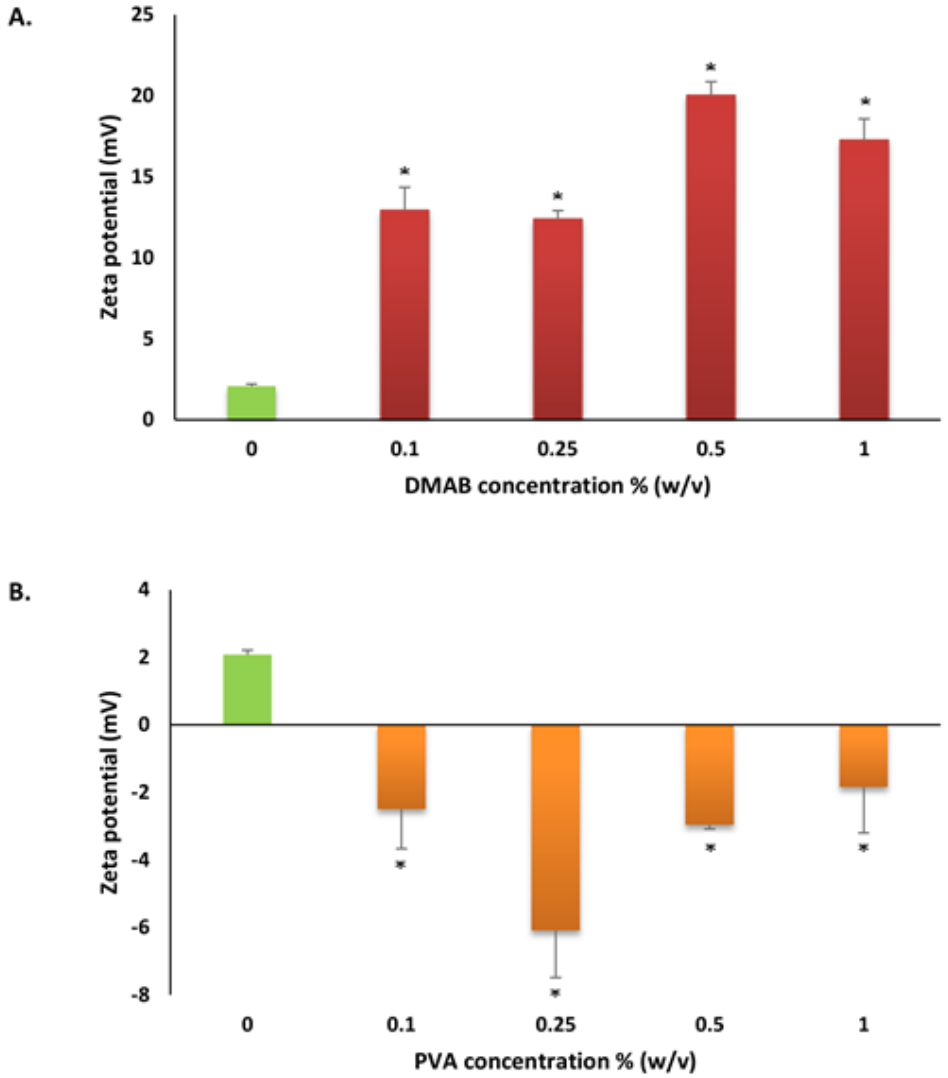


Fig. 4.1 Zeta potential measurements of A) DMAB and B) PVA formulated NPs of celecoxib. Values are expressed as mean ± SD, n=3. *p<0.05, significantly different from plain formulation

DMAB formulated CEL loaded NPs reached a peak zeta potential of 20.03 ± 0.84 mV at 0.5% w/v concentration. The anionic characteristics of PVA lead to the formation of NPs with slightly negative surface charges (Fig. 4.1B). PVA formulated NPs reached a peak zeta potential of -6.09 ± 1.39 mV with

0.25% w/v concentration.

Table 4.1
NP formulation with varying concentrations of DMAB or PVA

Formulation Number	Ingredients							
	Ethyl acetate (mL)	Water (mL)	DMAB (% w/v)	PVA (% w/v)	PLGA (mg)	Acetone (µL)	Celecoxib(mg)	Lecithin (mg)
1*	3	6	-	-	50	500	20	30
2	3	6	0.1	-	50	500	20	30
3	3	6	0.25	-	50	500	20	30
4	3	6	0.5	-	50	500	20	30
5	3	6	1	-	50	500	20	30
6	3	6	-	0.1	50	500	20	30
7	3	6	-	0.25	50	500	20	30
8	3	6	-	0.5	50	500	20	30
9	3	6	-	1	50	500	20	30

* Stabilizer free (plain) formulation

When comparing zeta potential as a measure of stability, all CEL-NP formulations containing DMAB or PVA showed significant alterations in NP system stability compared to stabilizer free formulations (plain formulation) (Fig. 4.1A and Fig. 4.1B). These results are indicative of altered NP characteristics as a result of adsorption or inclusion of DMAB and PVA onto or within the NP polymer shell. The inclusion of cationic and anionic DMAB (Fig. 4.1A) or PVA (Fig. 4.1B) on NP surfaces can effectively alter overall NP charge, in turn, effecting overall system stability [39-41].

In comparison to plain formulation, a significant reduction in particle size was seen in formulations incorporating 0.1%, 0.25%, and 0.5% DMAB (Fig. 4.2).

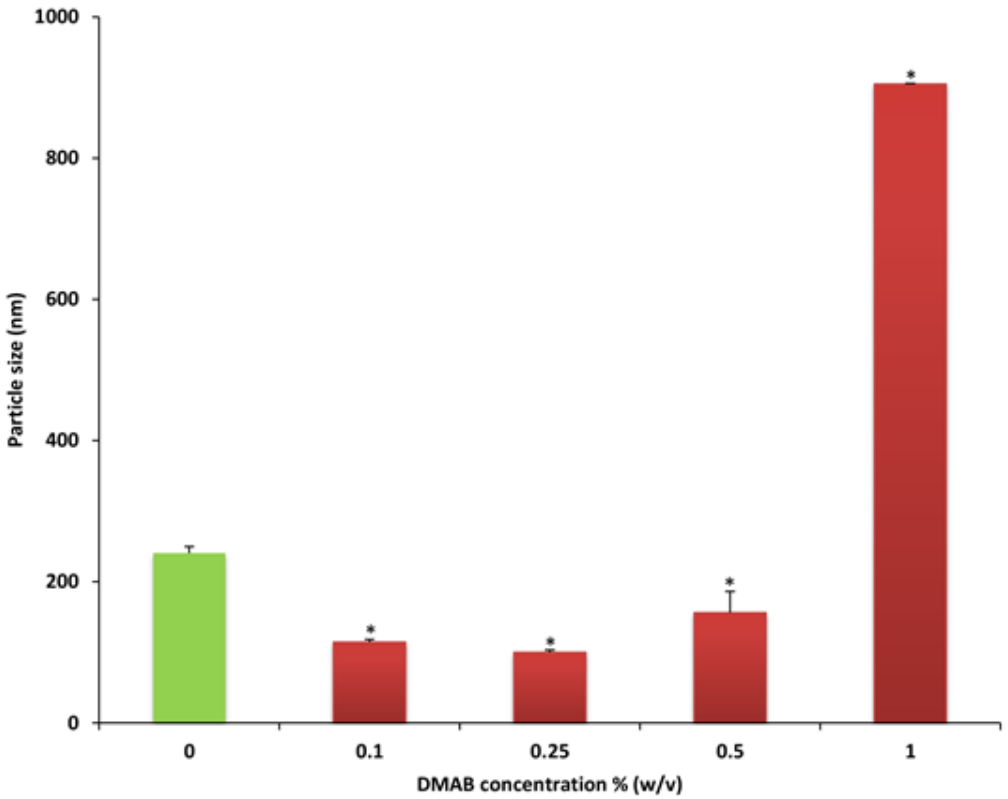


Fig. 4.2 Particle size analysis of increasing concentrations of DMAB compared to formulation without stabilizer (plain formulation). Values are expressed as mean ± SD, n=3. *p<0.05, significantly different from plain formulation

Particle size was significantly increased in 1% DMAB concentrations when compared to plain formulation. The largest reduction in particle size was achieved using 0.25% DMAB concentration (99.97 ± 3.27 nm) ($p < 0.01$). High concentrations of DMAB have been shown to increase solution viscosity, resulting in a direct increase in particle size [42], which may explain the significant rise in particle size noticed with CEL-NPs formulated using larger amounts of stabilizer. Furthermore, DMAB can act as a solubilizing agent for known hydrophobic compounds [36]. It is possible that lower DMAB concentrations may act to effectively reduce drug crystallization, further reducing NP size, which may explain NP size reductions seen in our study with lower DMAB concentrations (Fig. 4.2). Conversely, a significant increase in CEL solubility brought forth by higher DMAB content could function to increase NP drug loading capacitance and increase particle size by means of expanding NP drug content within the polymer shell.

Formulations using 0.1% w/v PVA did not demonstrate any significant difference in particle size when compared to plain formulation ($p > 0.77$). Particle size measurements of 0.25%, 0.5% and 1% PVA formulations were not detectable by our NICOMP particle sizer due to reduced entrapment efficiency and total drug concentrations in PVA based NP solution.

The amount (1.99 ± 0.01 mg) and percent (9.94 ± 0.01%) of CEL entrapped in formulations without stabilizer were compared to DMAB and PVA based formulations (Table 4.2). All stabilizer based formulations demonstrated significant changes in entrapment efficiency when compared to plain formulation (Table 4.2) ($P < 0.01$).

Table 4.2 Effects of stabilizer concentrations on celecoxib entrapment.

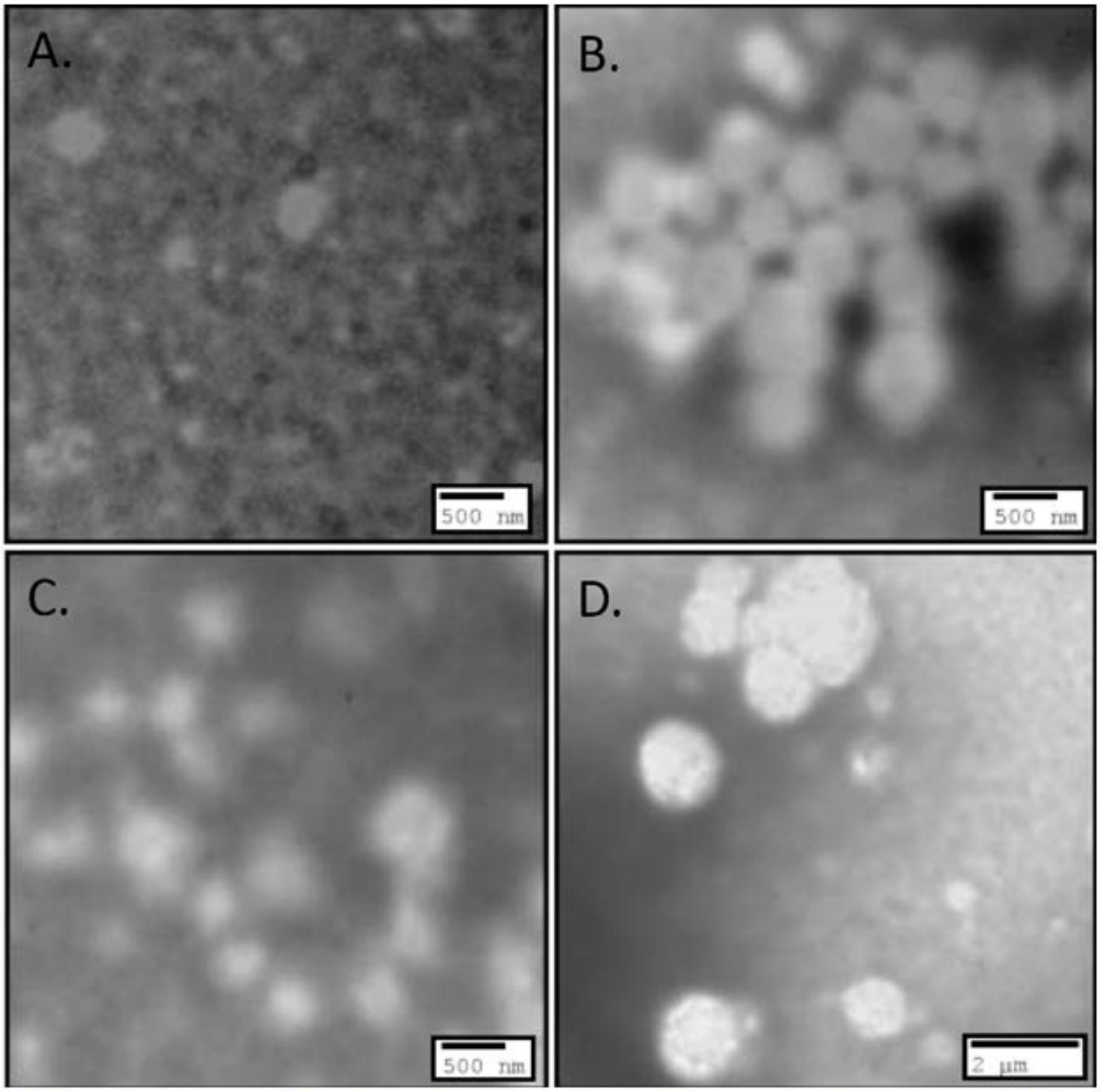
Stabilizer	Conc. (% w/v)	AE (mg)	% EE
Stabilizer free	0	1.99 ± 0.01	9.94 ± 0.01
DMAB	0.1	3.78 ± 0.01	18.85 ± 0.07*
	0.25	9.94 ± 0.08	49.70 ± 0.38*
	0.5	6.16 ± 0.01	30.84 ± 0.04*
	1	12.22 ± 0.01	61.07 ± 0.06*
PVA	0.1	9.23 ± 0.03	46.19 ± 0.16*
	0.25	0.11 ± 0.02	0.56 ± 0.03*
	0.5	0.06 ± 0.01	0.33 ± 0.02*
	1	0.66 ± 0.03	3.28 ± 0.14*

All values reported as mean ± SD (n = 3). Amount entrapped (AE) per 20 mg celecoxib. EE is the entrapment efficiency. *$P < 0.01$ compared to plain formulation.

All DMAB formulations and 0.1% PVA formulation exhibited significant increases in the level of CEL entrapment with a maximum percent entrapment of 61.07 ± 0.06% reached with 1% DMAB formulation. All PVA concentrations above 0.1% w/v underwent a significant reduction in drug entrapment (Table 4.2). The reduction in drug entrapment can be explained by elucidation of PVA properties. PVA is a highly hydrophilic stabilizer, which can result in reduced NP stability in aqueous solutions [43]. As PVA concentrations increase, the hydrophilic nature of the NP system increases. The increased inclusion of PVA into the NP polymer shell could increase hydrophilic properties leading to NP solubilization in the aqueous medium following organic phase evaporation. The increased

hydrophilic properties of PVA-NP systems could reduce entrapment and drug solubility leading to an increased loss of drug in solution precipitant following centrifugation.

In this study, DMAB was shown to effectively increase zeta potential, reduce particle size, and facilitate drug entrapment when compared to PVA based formulations. As such, DMAB based NP morphology was visualized and confirmed under transmission electron microscopy (TEM) (Fig. S1) with further variable analysis carried out using DMAB formulations.



S1 Figure TEM images of emulsifier based formulation illustrating morphology of A) 0.1% w/v DMAB formulated NPs, B) 0.25% w/v DMAB formulated NPs, C) 0.5% w/v DMAB formulated NPs, and D) 1% w/v DMAB formulated NPs.

0.28. ***Analysis of NP characteristics in absence of emulsifier***

To analyze effect of emulsifier on CEL loaded NP characteristics, formulations containing 0.1%, 0.25%, 0.5%, and 1% w/v DMAB without lecithin were developed, characterized and compared to previously observed characteristics of NP formulations with lecithin (Fig. 4.3).

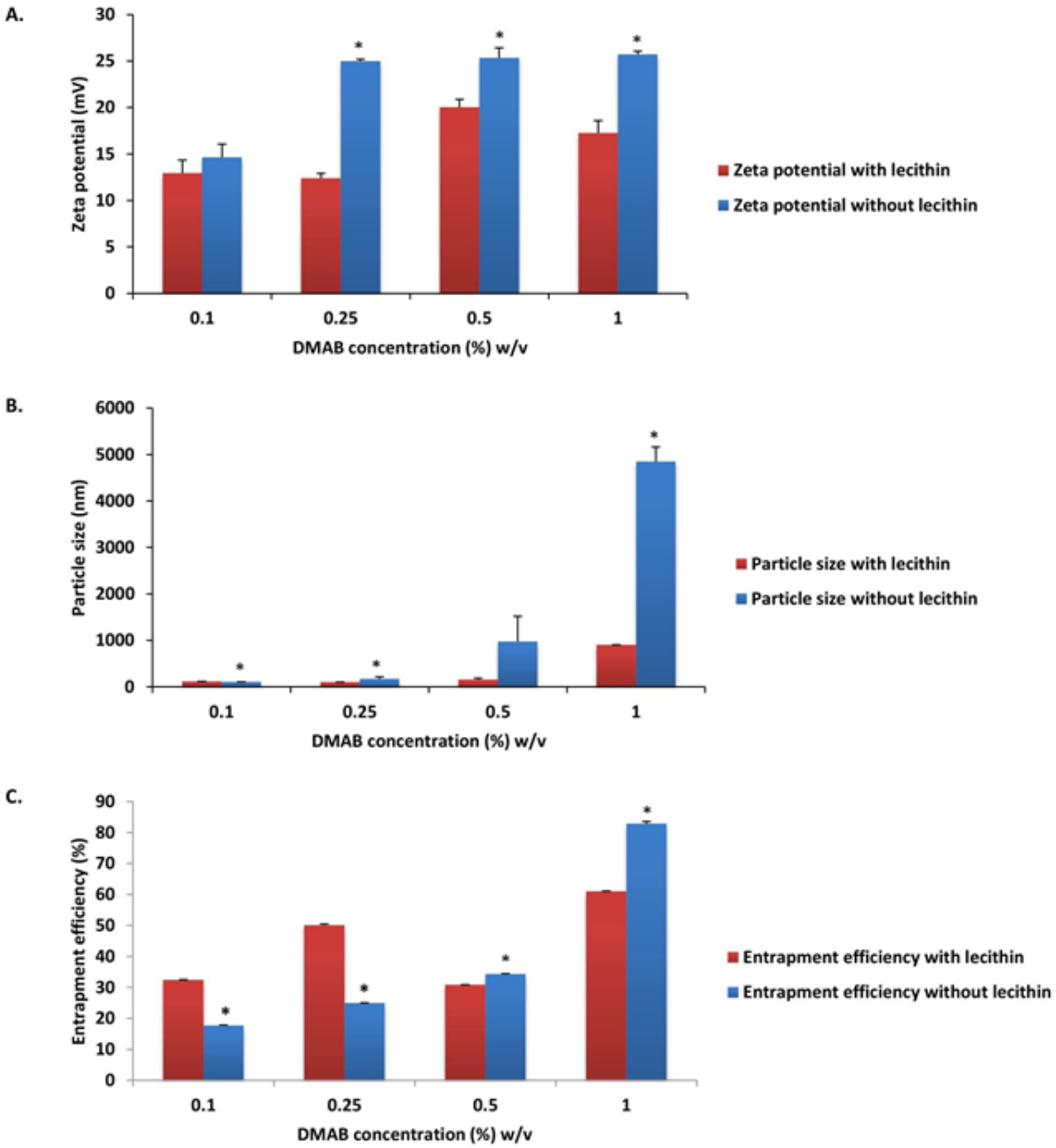
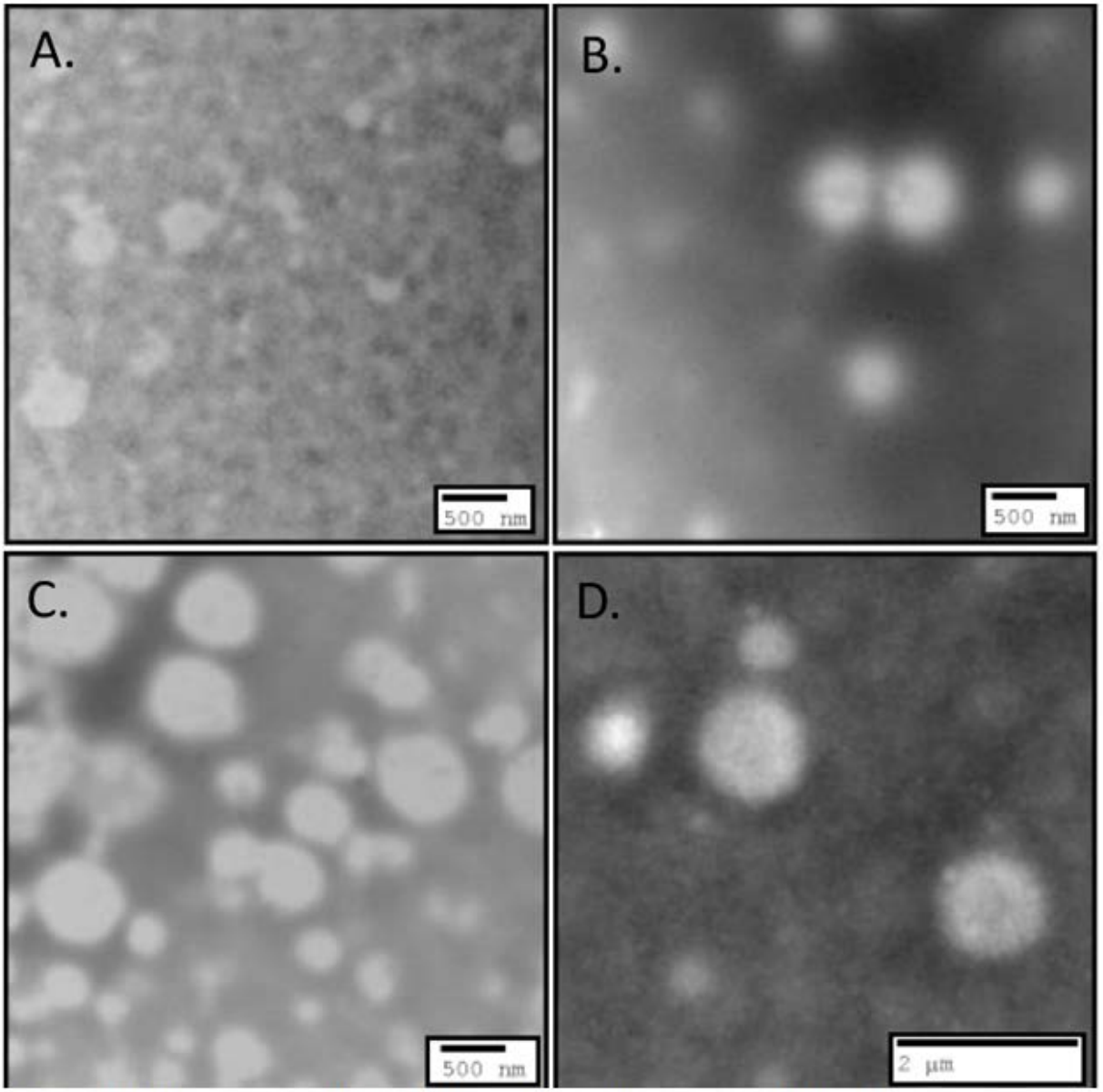


Fig. 4.3 Nanoparticle characteristic comparison of A) zeta potential, B) particle size, and C) entrapment efficiency of initial emulsifier based DMAB formulations with emulsifier free DMAB formulations. Values are expressed as mean ± SD, n=3. $p<0.05$, significantly different from initial formulations

NP

visual identification of emulsifier free formulations was performed via TEM analysis (Fig. S2).



S2 Figure TEM images of emulsifier free formulations illustrating morphology of 0.1% w/v DMAB formulated NPs, B) 0.25% w/v DMAB formulated NPs, C) 0.5% w/v DMAB formulated NPs, and D) 1% w/v DMAB formulated NPs.

When compared to emulsifier based formulations, absence of emulsifier resulted in a significant increase of zeta potential in formulations using 0.25%, 0.5%, and 1% stabilizer concentrations (Fig. 4.3A) ($P < 0.01$). These findings indicate that the use of an emulsifier may function to reduce overall particle repulsion and system stability. The cationic property of DMAB has become increasingly popular for

development of positively charged NPs [44]. In emulsifier free formulations, we found that rising zeta potential was associated with increased DMAB concentration. These results can be indicative of enhanced DMAB inclusion into the NP polymer shell. Furthermore, lecithin contains low concentrations of phosphatidic acid. The presence of phosphatidic acid can impart negatively charged, anionic characteristics during inclusion into NP formulations [45]. As such, the anionic properties of lecithin can act to reduce polymer surface charge and effectively mask the cationic charge associated with DMAB inclusion, which would explain the findings of reduced particle charge seen in emulsifier based NP formulations.

In emulsifier free formulations particle size increased with increasing stabilizer concentrations, with peak particle size reaching micron levels at 0.5% and 1% DMAB concentration (972.93 ± 547.71 nm and 4849.77 ± 313.75 nm, respectively) (Fig. 4.3B). These results indicate that lecithin effectively functions to reduce interfacial tension between organic and aqueous phases. In solvent evaporation processes, when organic phase is added to aqueous phase in a drop wise manner, the resultant organic droplets are stabilized by polymers formed at solute interfaces [45]. The type of polymer, surfactant, or emulsifier used can act to alter interfacial tensions between the organic droplets and the aqueous solution. After placement of organic phase into aqueous phase, interfacial spreading occurs as a result of diffusion between solvents, providing energy for NP formation [45]. NP size is dependent on rate of diffusion and diffusion is dependent on changes in the interfacial tension between organic and aqueous phases. Lower interfacial tension equates to smaller NP size properties [45-48]. The addition of compounds such as lecithin act to effectively change interfacial tension which can have altering effects on particle size and NP formation [48,49]. Changes in organic and aqueous phase interface alters the rate of solvent diffusion, as lecithin favors a higher organic phase to aqueous phase interface resulting in reduced particle size [46] as seen in our study.

Peak drug entrapment was seen at DMAB concentrations of 1% for formulations without emulsifier (82.91 ± 0.67%) (Fig. 4.3C). In relations to 1% DMAB formulation carried out with emulsifier, these results equate to an almost 22% increase in NP drug loading ($P < 0.01$). In theory, inclusion of lecithin could act to offset surface tension allowing for fast organic phase diffusion into the aqueous phase [50]. The alteration in interfacial tension could also function to reduce barrier transport of drug outside of the organic phase during solvent diffusion. Inclusion of lecithin into the polymeric shell with increasing concentrations of DMAB resulted in a net reduction in drug entrapment compared to its emulsifier free counterpart. Much like lecithin, DMAB can form micelle aggregates that function through hydrophobic interactions of DMAB with the hydrophobic core of the NP [34]. The interactions of the hydrophobic portion of the stabilizer can function to solubilize the hydrophobic drug entrapped within the NP core [36]. It is possible that as concentration of both DMAB and lecithin increased in formulations, the net rise in hydrophobic interaction resulted in increased NP and CEL solubility leading to drug leakage and reduced drug entrapment. Similar results were obtained by Thakkar *et al.* when using Span-85 as an emulsifying agent during the development of CEL microspheres [51]. In the study, it was found that addition of emulsifier with high concentrations of stabilizer (1% w/v) enhanced CEL solubility and dissolution, which led to a reduction in both particle size and drug entrapment.

Previously, our lab completed formulation of diclofenac (a non-selective NSAID) loaded PLGA-NPs using DMAB and PVA [34]. With no change in drug amount (45 mg) or use of emulsifier, diclofenac loaded PLGA-NPs with DMAB or PVA exhibited negative surface charges and a peak entrapment efficiency as high as 80.21 ± 1.21%. The negative NP surface charge associated with diclofenac NP formulations using DMAB contrast with the highly positive surface charge of DMAB formulated CEL loaded NPs found in this study. When using diclofenac, PVA formulated NPs showed smaller negative surface charges, similar to the negative surface charge characteristics associated with our PVA formulated CEL-NP formulation. In physiological conditions, diclofenac is a negatively charged

molecule which may play a role in the development of negatively charged NPs during formulation with DMAB [52]. Conversely, at physiological pH, CEL presents as a neutrally charged molecule [53] that, when formulated with cationic DMAB, resulted in formation of positively charged NPs. Particle size analysis showed a similar pattern when comparing CEL formulation results with that of diclofenac. Diclofenac NP formulation showed a maximum increase of NP size (189.9 ± 4.9 nm) using 1% w/v DMAB. Similarly, results of our CEL formulation study demonstrated maximum NP size with 1% w/v DMAB concentration (Fig. 4.3B). Measurements of entrapment efficiency showed opposite effects. For diclofenac NPs, a linear reduction in total entrapment efficiency was seen with regards to increasing DMAB concentrations with the lowest amount of diclofenac entrapment occurring with 1% w/v DMAB. Conversely, maximum CEL loading seen within this study was observed at 1% DMAB, which when compared to the highly polarizable diclofenac [54], supports the theory that higher concentrations of DMAB may increase solubility of lipophilic drugs such as CEL, in turn leading to increases in particle size and drug entrapment [42].

0.28. ***Effect of drug amount on NP characteristics***

To study the effect of drug amount on NP characteristics, formulations consisting of 0.25% DMAB concentrations were chosen based on their sufficient size and general representation of drug entrapment and zeta potential. In conjunction with previously formulated NP systems using 20 mg CEL, new NPs with or without emulsifier were formulated with increasing amounts (5, 10, and 15 mg) of CEL (Table 4.3).

Table 4.3
Preparation method for NP formulations with differing drug amounts

Formulation Number	Ingredients						
	Ethyl acetate (mL)	Water (mL)	DMAB (% w/v)	PLGA (mg)	Acetone (µL)	Celecoxib (mg)	Lecithin (mg)
1	3	6	0.25	50	500	20	30
2	3	6	0.25	50	500	15	30
3	3	6	0.25	50	500	10	30
4	3	6	0.25	50	500	5	30
5	3	6	0.25	50	500	20	-
6	3	6	0.25	50	500	15	-
7	3	6	0.25	50	500	10	-
8	3	6	0.25	50	500	5	-

Morphological characterization of NPs formulated with (Fig. 4.4) and without (Fig. 4.5) emulsifier at various drug amounts showed spherical shape and size similar to what was noticed in previous formulation studies [24,25,55-59]. All NP formulations without emulsifier displayed significantly higher zeta potential compared to formulations with emulsifier (Fig. 4.6A) ($p < 0.01$). Maximum zeta potential was reached in formulations of 20 mg CEL without emulsifier (25.00 ± 0.18 mV). Formulations with emulsifier reached peak zeta potential using 15 mg CEL (17.15 ± 0.36 mV). These results further indicate that use of emulsifiers such as lecithin, can function to mask surface charge of the incorporated stabilizer thereby reducing overall cationic charge associated with DMAB formulated NPs [45].

Peak size reduction and entrapment efficiency for formulations with (70.87 ± 1.24 nm and 95.55 ± 0.66%, respectively) and without (92.97 ± 0.53 nm and 95.93 ± 0.27%, respectively) emulsifier was achieved using 5 mg drug amounts (Fig. 4.6B and Fig. 4.6C, respectively). These results indicate an important role for drug solubility on the characterization of CEL loaded NPs.

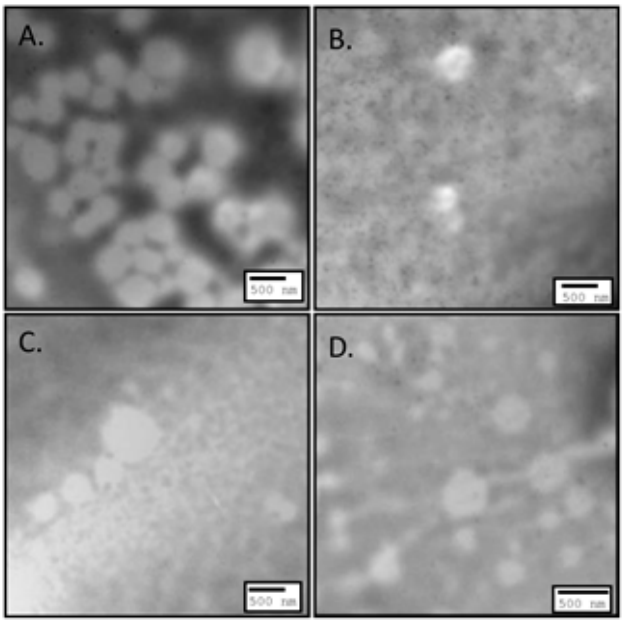


Fig. 4.4 TEM images illustrating morphology of 0.25% w/v DMAB NP formulations with emulsifier at A) 5 mg drug amount, B) 10 mg drug amount, C) 15 mg drug amount, and D) 20 mg drug amount.

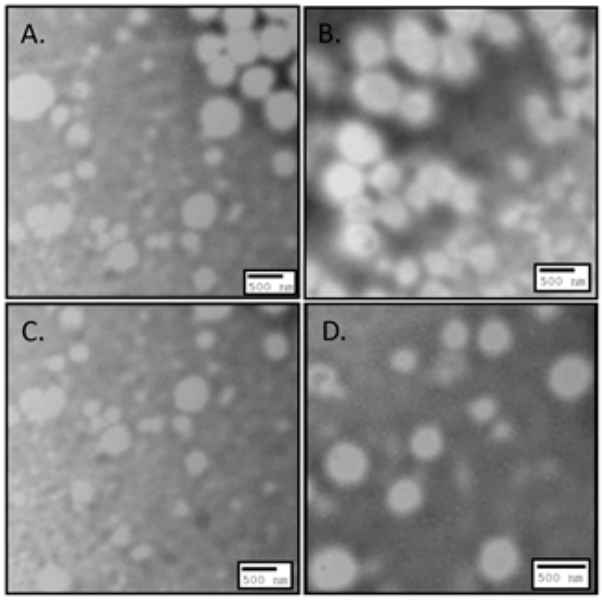


Fig. 4.5 TEM images illustrating morphology of 0.25% w/v DMAB NP formulations without emulsifier at A) 5 mg drug amount, B) 10 mg drug amount, C) 15 mg drug amount, and D) 20 mg drug amount.

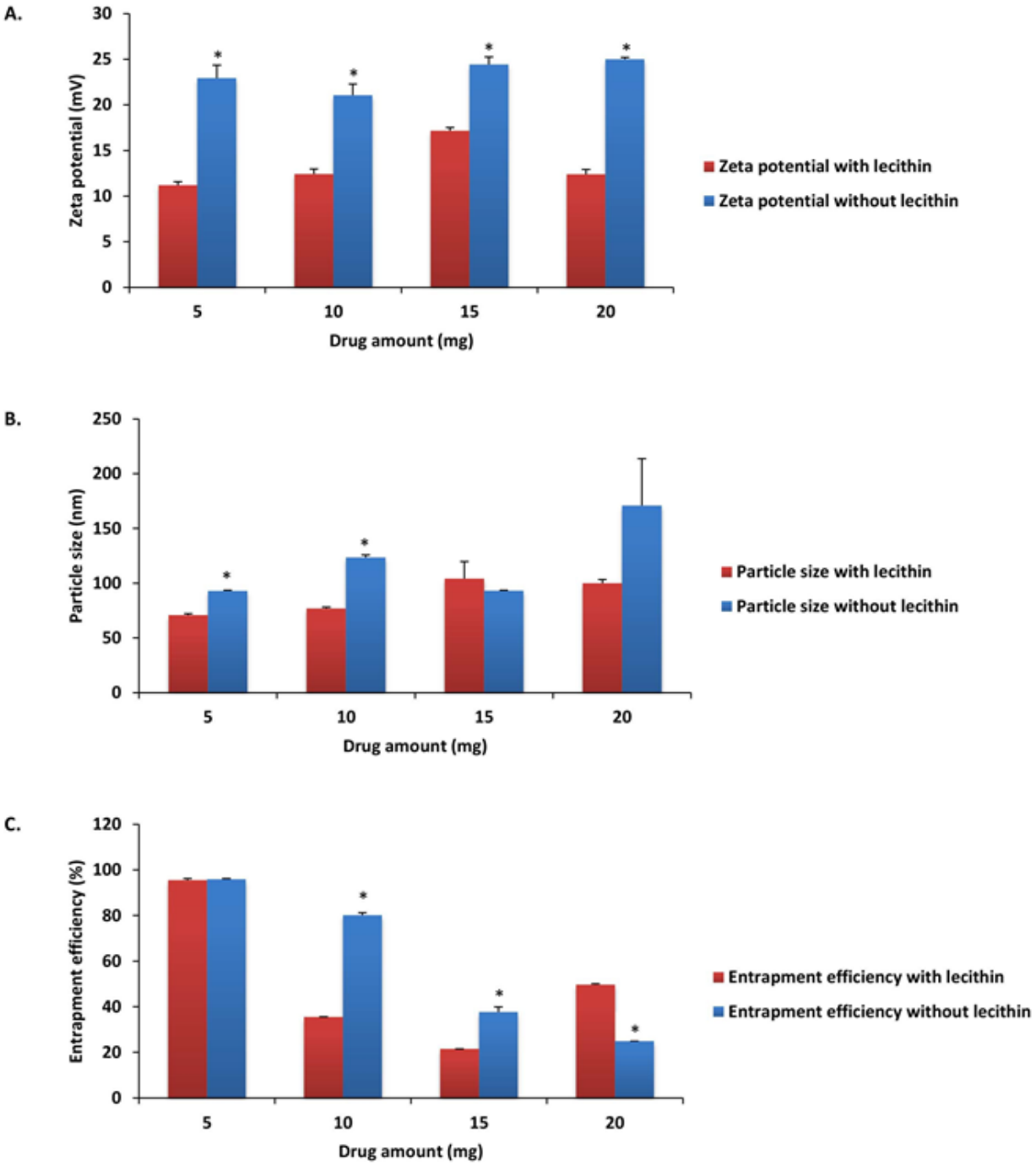


Fig. 46 The effect of varying drug amounts on A) zeta potential, B) NP size, and C) entrapment efficiency. Values are expressed as mean ± SD, n=3. $p<0.05$, significantly different from formulations with emulsifier.

CEL belongs to the biopharmaceutical classification system class II drug exhibiting poor aqueous solubility [60-62]. During formulation, several techniques such as size reduction, use of emulsifier, or surfactants can be applied to help increase the degree of drug solubility in aqueous media [63]. In

applications towards NP production, several of these techniques are often applied in order to increase drug solubility and prevent drug precipitation outside of the NP shell. In this study, stabilizers and an emulsifier were used to measure effects on particle characteristics through means of increased solubility. The use of drug amount was also analyzed as a measurement of solubility effects on zeta potential, particle size, and drug entrapment. In an effort to optimize drug entrapment, drug amounts were titrated to measure extent of effects on NP encapsulation. The solubility of a drug is related to surface area and volume ratios [63]. In particle size reduction, surface area is increased and allows greater interactions with the solvent which causes an increase in solubility. In conjunction with particle size reduction via sonication, reductions in drug amount allows further enhancement in surface area to volume ratio. We found that when drug amount was decreased in CEL-NP formulations, entrapment efficiency was able to achieve over 95% loading capacitance (Fig. 4.6C). This success indicates the importance of drug amount in conjunction with size reduction for the prevention of drug precipitation and enhancement of drug entrapment efficiency during NP formulations.

Interestingly, in this study it was found that as drug amount increased to 20 mg, total entrapment of CEL increased in formulations with emulsifier while amounts of 15 mg and 10 mg CEL displayed increasing total drug entrapment in regards to emulsifier free formulation (Fig. 4.6C). It is possible that higher concentrations of CEL undergoes enhanced solubilization in the presence of emulsifier, enabling a larger degree of drug entrapment in the presence of higher drug amounts. Furthermore, lecithin is a non-ionic emulsifier known to impart steric stabilizing effects in colloidal systems, preventing particle collision and reducing drug leakage [64,65]. It is possible that as drug amounts increase, lecithin functions to increase drug solubility, stabilize NP formation, and reduce drug leakage leading to an increase in drug entrapment. The observation that larger drug amounts undergo increased NP entrapment in the presence of lecithin may support the use of emulsifiers during NP production of high, lipophilic drug concentrations.

3.4. Stability of CEL loaded PLGA-NPs

To avoid particle aggregation and coalescence, it is recommended to store PLGA-NP systems at 4 °C [66]. Therefore, to analyze stability of PLGA-NP systems, emulsions of varying drug amounts with or without emulsifier were kept at 4 °C for a period of 16 weeks then characterized to determine storage effects on zeta potential, particle size, and drug entrapment efficiency.

Results showed that zeta potential, particle size, and entrapment efficiency were at or below initial reported NP characterization measurements (Fig. 4.7 and Fig. 4.8).

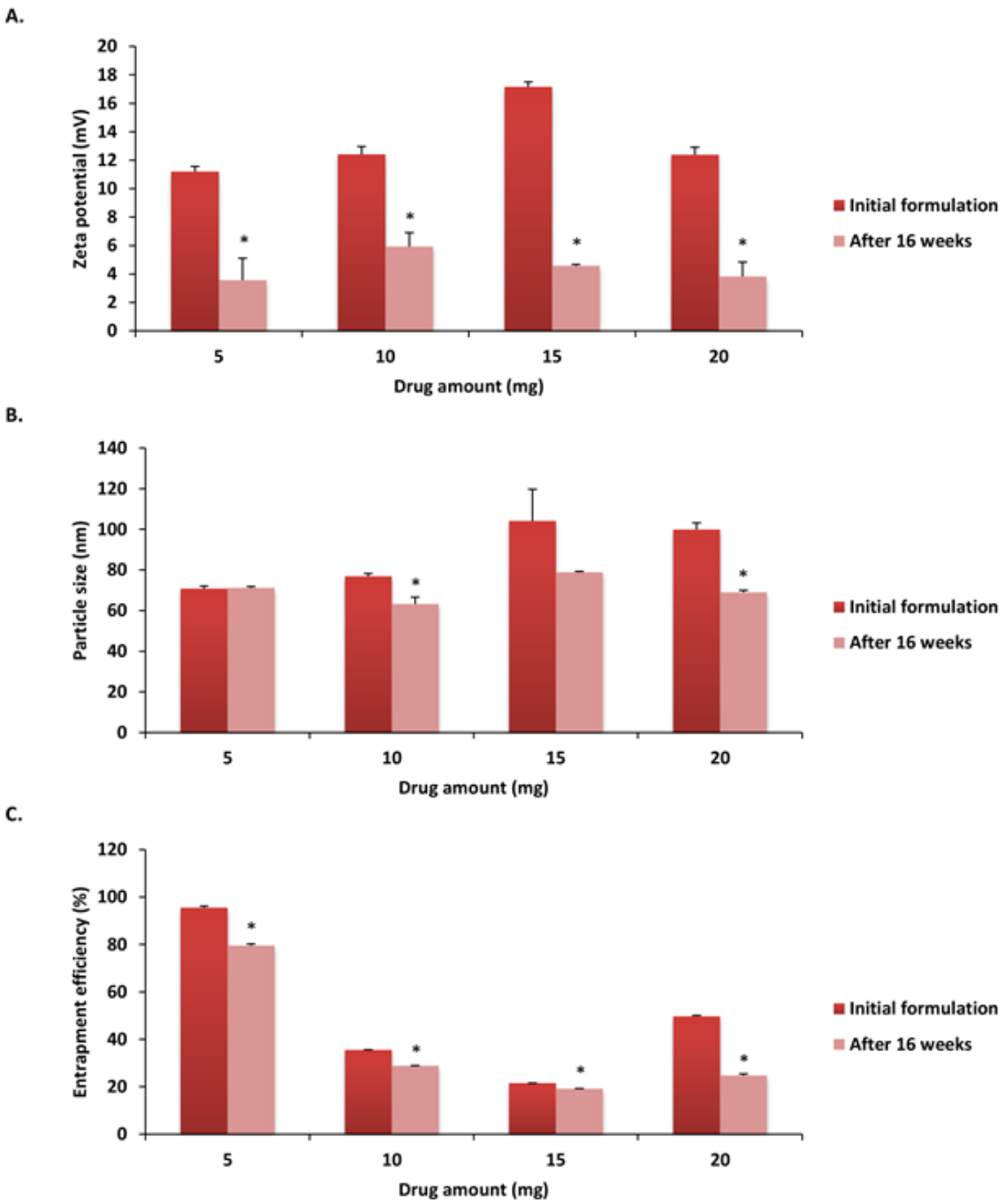


Fig. 4.7 Nanoparticle characteristic comparison of A) zeta potential, B) particle size, and C) entrapment efficiency of initial emulsifier based formulations with those observed following 16 weeks cold storage at 4°C. Values are expressed as mean ± SD, n53. *$p<0.05$, significantly different from initial formulation

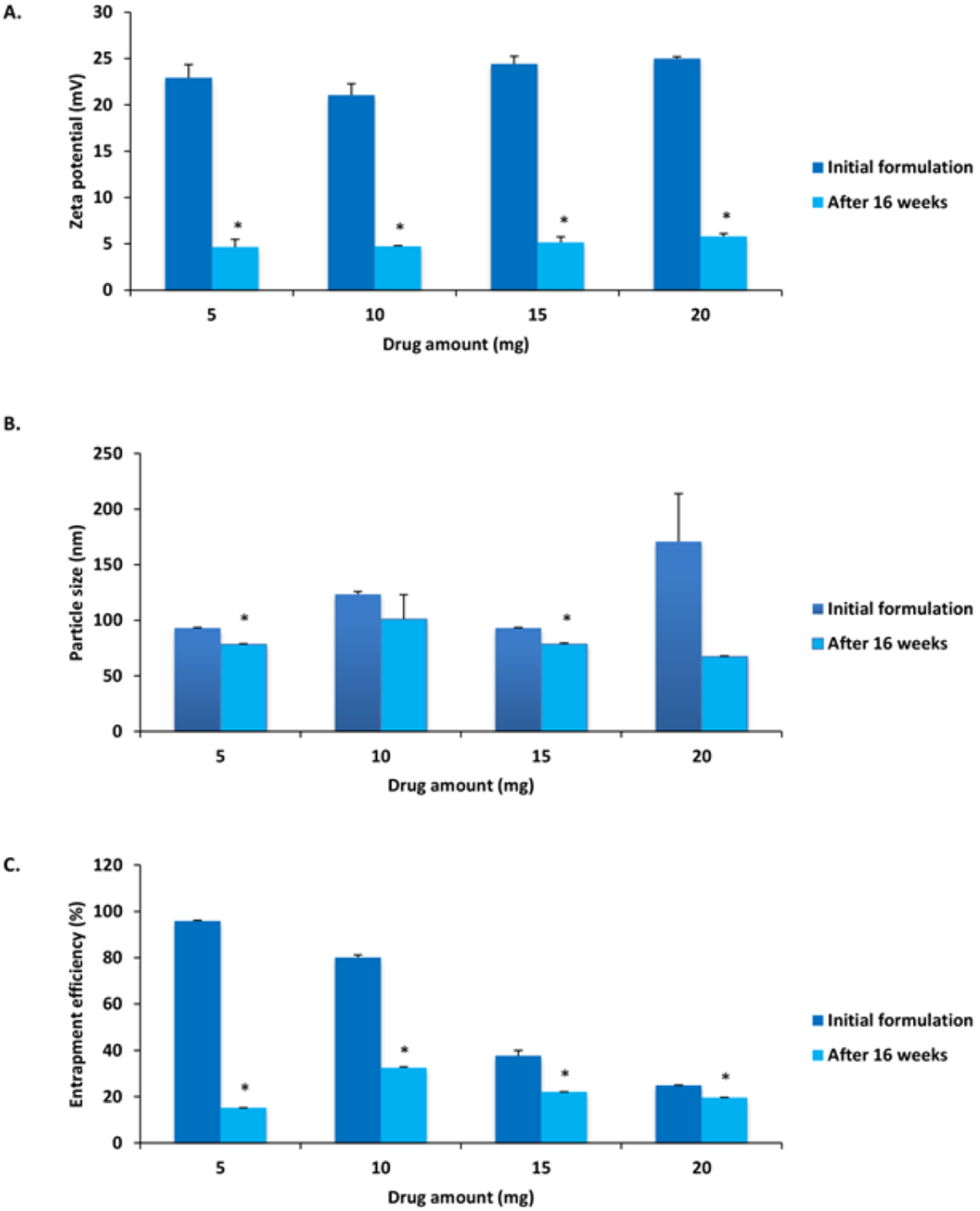


Fig. 4.8 Nanoparticle characteristic comparison of A) zeta potential, B) particle size, and C) entrapment efficiency of initial emulsifier free formulations with those observed following 16 weeks cold storage at 4°C. Values are expressed as mean ± SD, n=3. *$p<0.05$, significantly different from initial formulations

All peak characteristic measurements after 16 weeks of cold storage was noticed in formulations that included emulsifier (Fig. 4.7). When compared to our initial formulations (Fig. 4.6), zeta potential was

reduced across all formulations ($p < 0.05$), with a peak zeta potential seen in formulations using 10 mg drug amounts with emulsifier (5.92 ± 0.98 mV) (Fig. 4.7A). When analyzing particle diameter, a peak reduction was seen in 10 mg formulations with emulsifier (63.23 ± 3.33 nm). Furthermore, when compared to initial characteristic measurements, significant particle size reductions were seen in 10 mg and 20 mg CEL formulations with emulsifier (Fig. 4.7B) ($p < 0.01$), as well as 5 mg and 15 mg formulations without emulsifier (Fig. 4.8B) ($p < 0.01$). All formulations showed a significant reduction ($p < 0.01$) in entrapment efficiency with the highest level of entrapment maintained in 5 mg formulation with emulsifier (79.58 ± 0.611%) (Fig. 4.7C). These results indicate the possible role emulsifying agents may have on maintenance of NP stability. The reduction of zeta potential observed in all formulations could be a result of possible DMAB dissociation from the NP shell after 16 weeks. Loss of DMAB would lead to reduced particle charge, net repulsion, and stability resulting in increased drug leakage, particle size reduction, and reduced entrapment efficiency [67]. Furthermore, the emulsifier in our formulation may be exerting unknown effects on drug permeation and NP aggregation, allowing for enhanced time-dependent stability of PLGA-NPs formulated with lecithin [68].

Conclusion

In this study, we performed a solvent evaporation technique to developed and characterize CEL loaded PLGA-NPs using varying concentrations of DMAB or PVA as stabilizer. NPs were examined and characterized based on zeta potential, size, drug entrapment efficiency, and morphology. The results of this study showed that the use of DMAB as stabilizer led to the development of NPs that displayed sufficient size and stability with moderate increases in drug entrapment when compared to plain formulation. Of the two stabilizers, DMAB proved to be highly efficient in developing well characterized CEL loaded PLGA based NPs, whereas PVA based formulations failed to reach optimum parameters in NP development. Variables such as emulsifier and drug amount were also analyzed to

further optimize NP formulations. When formulations were carried out in the presence of emulsifier a reduction in zeta potential was noted. Emulsifier based formulations displayed reduced surface charge as a consequence of lecithin induced anionic interactions and masking of cationic DMAB properties, indicating that in the presence of DMAB based formulations, emulsifiers such as lecithin may act to reduce NP stability and formula optimization. Additional formula evaluation showed that reduction in drug amount was effective at reducing particle size and enhancing drug entrapment efficiency, further elucidating the role of drug solubility and the importance of increasing surface area to solvent interactions for effective development of CEL loaded NPs. Interestingly, while use of emulsifier resulted in reduced zeta potential and system stability, time-dependent stability testing which looked at zeta potential, size, and entrapment efficiency after 16 weeks cold storage showed peak particle characteristics in formulations with emulsifier. These results may indicate that while emulsifiers such as lecithin function to reduce overall particle charge during formulation, they could function to prolong NP system stability over an extended period of time. However, further testing is needed to determine the extent of emulsifier effects on CEL loaded PLGA-NP stability. Overall, results of our study indicate formulation of PLGA-NPs using 0.25% w/v DMAB and 5 mg CEL amounts without emulsifier for creation of highly entrapped, stable NPs of a sufficient size that could function to enhance the application of orally delivered CEL and provide a potential effective dosage form for CEL administration.

Supporting Information

Figure S1. TEM images of emulsifier based formulation illustrating morphology of A) 0.1% w/v DMAB formulated NPs, B) 0.25% w/v DMAB formulated NPs, C) 0.5% w/v DMAB formulated NPs and D) 1% w/v DMAB formulated NPs.

Figure S2. TEM images of emulsifier free formulations illustrating morphology of A) 0.1% w/v DMAB formulated NPs, B) 0.25% w/v DMAB formulated NPs, C) 0.5% w/v DMAB formulated NPs and D) 1% w/v DMAB formulated NPs.

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Figure Legends

Figure 4.1. Zeta potential measurements of A) DMAB and B) PVA formulated NPs of celecoxib. Values are expressed as mean ± SD, n = 3. $^{*}p < 0.05$, significantly different from plain formulation.

Figure 4.2. Particle size analysis of increasing concentrations of DMAB compared to formulation without stabilizer (plain formulation). Values are expressed as mean ± SD, n = 3. $^{*}p< 0.05$, significantly different from plain formulation.

Figure 4.3. Nanoparticle characteristic comparison of A) zeta potential, B) particle size, and C) entrapment efficiency of initial emulsifier based DMAB formulations with emulsifier free DMAB formulations. Values are expressed as mean ± SD, n = 3. $^{*}p < 0.05$, significantly different from initial formulations.

Figure 4.4. TEM images illustrating morphology of 0.25% w/v DMAB NP formulations with emulsifier at A) 5 mg drug amount, B) 10 mg drug amount, C) 15 mg drug amount, and D) 20 mg drug amount.

Figure 4.5. TEM images illustrating morphology of 0.25% w/v DMAB NP formulations without emulsifier at A) 5 mg drug amount, B) 10 mg drug amount, C) 15 mg drug amount, and D) 20 mg drug amount

Figure 4.6. The effect of varying drug amounts on A) zeta potential, B) NP size, and C) entrapment efficiency. Values are expressed as mean ± SD, n = 3. $^{*}p < 0.05$, significantly different from formulations with emulsifier.

Figure 4.7. Nanoparticle characteristic comparison of A) zeta potential, B) particle size, and C) entrapment efficiency of initial emulsifier based formulations with those observed following 16 weeks cold storage at 4 °C. Values are expressed as mean ± SD, n = 3. $^{*}p < 0.05$, significantly different from initial formulations.

Figure 4.8. Nanoparticle characteristic comparison of A) zeta potential, B) particle size, and C) entrapment efficiency of initial emulsifier free formulations with those observed following 16 weeks cold storage at 4 °C. Values are expressed as mean ± SD, n = 3. $^{*}p < 0.05$, significantly different from initial formulations.

CHAPTER 5

SUMMARY

The purpose of this study was to formulate, develop and optimize a new NP based drug delivery system that could be used to enhance and/or mitigate effects of the nonsteroidal anti-inflammatory drugs (NSAIDs) diclofenac or celecoxib (Cooper and Harirforoosh 2014; Cooper and Harirforoosh 2014). A solvent-evaporation technique was used to developed and characterize diclofenac or celecoxib loaded polymeric NPs using DMAB or PVA as stabilizer. NPs were characterized based on size, stability, morphology and percent of drug entrapped. For all active pharmaceutical ingredients examined in this study, the use of DMAB as stabilizer resulted in the development of NP systems that demonstrated sufficient size and stability characteristics while also showing moderate increases in drug entrapment when compared to plain formulation. Optimization experiments were designed and carried out for each drug as well. Effects of centrifugal force, stabilizer concentration, emulsifier and total drug amounts on formulation parameters such as zeta potential, drug entrapment, and particle size were examined.

During formulation studies, it was found that PLGA based NPs using DMAB as stabilizer, resulted in formation of highly stable NPs for both diclofenac and celecoxib. In diclofenac formulation, PLGA based NPs showed the lowest particle size (108 ± 2.1 nm) and highest zeta potential (-27.71 ± 0.6 mV) at 0.1 and 0.25% DMAB respectively, when centrifuged at 12,000 rpm. Peak drug entrapment reached 77.3 ± 3.5% (20). Results of diclofenac and PVA based NP formulation showed the smallest particle size (92.4 ± 7.6 nm) and highest zeta potential (11.14 ± 0.5 mV) at 0.25% and 1% w/v, respectively, after centrifugation at 12,000 rpm. Diclofenac NP formulations utilizing PVA stabilizer reached an entrapment efficiency of 80.2 ± 1.2%. Over all, zeta potential levels were dramatically lower in PVA based formulations then DMAB based formulations. The lower level of zeta potential seen in PVA formulas indicates a high degree of system instability and particle agglomeration (Cooper, D.L. 2014). Hindrance of particle repulsion brought forth by the reduced surface charge would

allow cohesion and aggregation of NP presented within the system. This aggregation would negate the benefits of nanosizing and lead to leaching of the drug out of the NP shell and/or falling out of solution. This finding alone supports the use of DMAB as the primary stabilizer to be used during diclofenac NP formulation.

In optimization techniques it was found that both emulsifier and drug amount could affect NP characteristics during formulation of celecoxib loaded NPs (Cooper and Harirforoosh 2014). For these formulations, peak zeta potential was achieved using 15 mg celecoxib with emulsifier (17.15 ± 0.36 mV) and 20 mg celecoxib without emulsifier (25.00 ± 0.18 mV). The largest particle size reduction and entrapment efficiency was achieved using 5 mg celecoxib formulations with (70.87 ± 1.24 nm and 95.55 ± 0.66%, respectively) and without (92.97 ± 0.51 nm and 95.93 ± 0.27%, respectively) emulsifier. It is important to note that during celecoxib NP formula optimization, reaching entrapment efficiency at or above 50%, while maintaining favorable characteristic profiles for both zeta potential and particle size, was difficult to achieve. Initial formulations that looked at effects of stabilizer concentration and emulsifier on NP characteristics showed entrapment percent's over 50% only when total stabilizer concentrations reached 0.5 – 1%. Unfortunately, although entrapment efficiency reached adequate levels, higher amounts of stabilizer, both with and without emulsifier, demonstrated particle sizes on a micron scale. When dealing with nano based formulations, these findings are unacceptable. It was only when optimization techniques were employed that altered the actual amount of drug used, were favorable characteristics seen among the categories of both zeta potential and particle size as well as drug entrapment. Effects of drug amount on formulation characteristics were analyzed by formulating celecoxib based NP systems using 5 mg, 10 mg, 15 mg or 20 mg drug amounts. In this study, when lower amounts of drug was used (5 mg) entrapment efficiency reached a peak level of over 95% with or without the use of an emulsifier. Do to the extremely high levels of entrapment seen, a conclusion could be drawn that formulations performed with 5 mg drug amounts gave the best celecoxib loaded NPs.

Furthermore, this study shows that formulas utilizing 5 mg drug amounts without the use of a secondary emulsifier led to formation of sufficiently small NPs with a high degree of stability. These findings support the conclusion that formulation of celecoxib NPs using lower drug amounts without emulsifier results in optimal NP system development.

Overall, results of this study demonstrated successful formulation of diclofenac and celecoxib loaded polymeric NPs (Cooper and Harirforoosh 2014; Cooper and Harirforoosh 2014). The findings presented here may indicate a novel delivery method that could function to decrease or eliminated drug induced adverse side effects seen in the clinical settings (Marimuthu et al 2013; Tai et al 2013; Cooper and Harirforoosh 2014; Cooper and Harirforoosh 2014). However, while the present research is promising, further research is needed to elucidate the overall effect of NP drug delivery on these select pharmacotherapeutics.

Despite successful formulation techniques presented here, extensive research is still needed to uncover the exact affect these new formulations will have on overall drug function and characteristics. In depth research and examinations, including *in vivo* studies should be performed to measure the extent of NP effects on maintenance of drug efficacy and the formation of adverse events associated with acute and chronic drug exposure. For NSAID formulated NPs, *in vivo* pharmacokinetic and pharmacodynamic studies should be carried out to assess and examine drug dosing and formula effects on systemic exposure, onset of adverse effects and maintenance of overall drug efficacy. Due to known adverse effects of NSAIDs in regards to gastrointestinal disturbances and renal complications, the extent of drug induced NSAID NP effects to be analyzed should include analysis of electrolyte concentrations before and after regimen initiation, and gastrointestinal histological examination. Furthermore, biomarkers such as blood urea nitrogen and urinary kidney injury molecule 1, as a measure of the extent of drug induced renal stress, should also be examined. Examination of effects on inflammation and prostaglandin

expression as a measure of drug efficacy could be carried out utilizing ELISA assays and biomarker analysis of cyclo-oxygenase (COX), myeloperoxidase, and prostaglandin E2 levels.

The experiments outlined above are designed to evaluate the effectiveness of these polymer encapsulate drugs in achieving enhanced systemic absorption following oral dosing. Utilization of *in vivo* data will serve as a strong indicator in regards to formulation effectiveness at achieving adequate and sustained plasma drug concentrations and further elucidate effects of these formulations on associated adverse drug events and the maintenance or enhancement of overall drug efficacy.

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